



US009657053B2

(12) **United States Patent**  
**Thacher et al.**

(10) **Patent No.:** **US 9,657,053 B2**  
(45) **Date of Patent:** **\*May 23, 2017**

(54) **MODULATORS OF RETINOID-RELATED ORPHAN RECEPTOR GAMMA**

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(\* ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 350 days.  
  
This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/766,076**

(22) Filed: **Feb. 13, 2013**

(65) **Prior Publication Data**

US 2013/0150333 A1 Jun. 13, 2013

**Related U.S. Application Data**

(63) Continuation of application No. 13/114,616, filed on May 24, 2011, now Pat. No. 8,389,739, which is a continuation of application No. 11/867,637, filed on Oct. 4, 2007, now abandoned.

(60) Provisional application No. 60/849,903, filed on Oct. 5, 2006.

(51) **Int. Cl.**

**A61K 31/58** (2006.01)  
**A01N 41/06** (2006.01)  
**A01N 37/00** (2006.01)  
**C07J 9/00** (2006.01)  
**C07C 311/21** (2006.01)  
**C07D 401/14** (2006.01)  
**C07J 21/00** (2006.01)  
**C07D 249/12** (2006.01)  
**C07D 493/10** (2006.01)

(52) **U.S. Cl.**

CPC ..... **C07J 9/005** (2013.01); **A61K 31/58** (2013.01); **C07C 311/21** (2013.01); **C07D 249/12** (2013.01); **C07D 401/14** (2013.01); **C07D 493/10** (2013.01); **C07J 9/00** (2013.01); **C07J 21/00** (2013.01)

(58) **Field of Classification Search**

None  
See application file for complete search history.

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(57) **ABSTRACT**

Methods for modulating (inhibiting or stimulating) retinoid-related orphan receptor  $\gamma$  (ROR $\gamma$ ) activity. This modulation has numerous effects, including inhibition of T<sub>H</sub>-17 cell function and/or T<sub>H</sub>-17 cell activity, and inhibition of re-stimulation of T<sub>H</sub>-17 cells, which are beneficial to treatment of inflammation and autoimmune disorders. Stimulation of ROR $\gamma$  results in stimulation of T<sub>H</sub>-17 cell function and/or activity which is beneficial for immune-enhancing compositions (e.g., vaccines).

**28 Claims, 21 Drawing Sheets**

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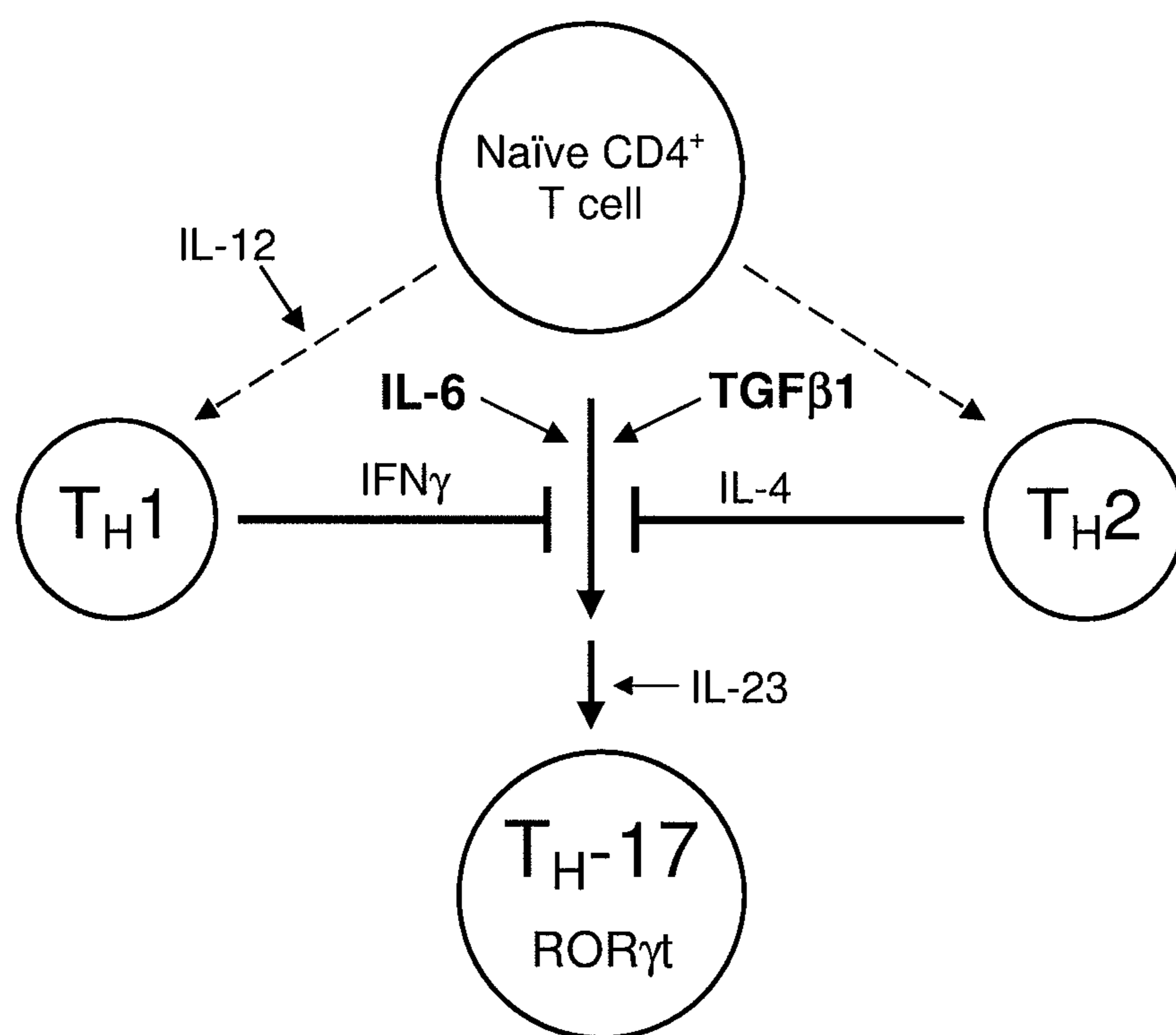


FIGURE 1

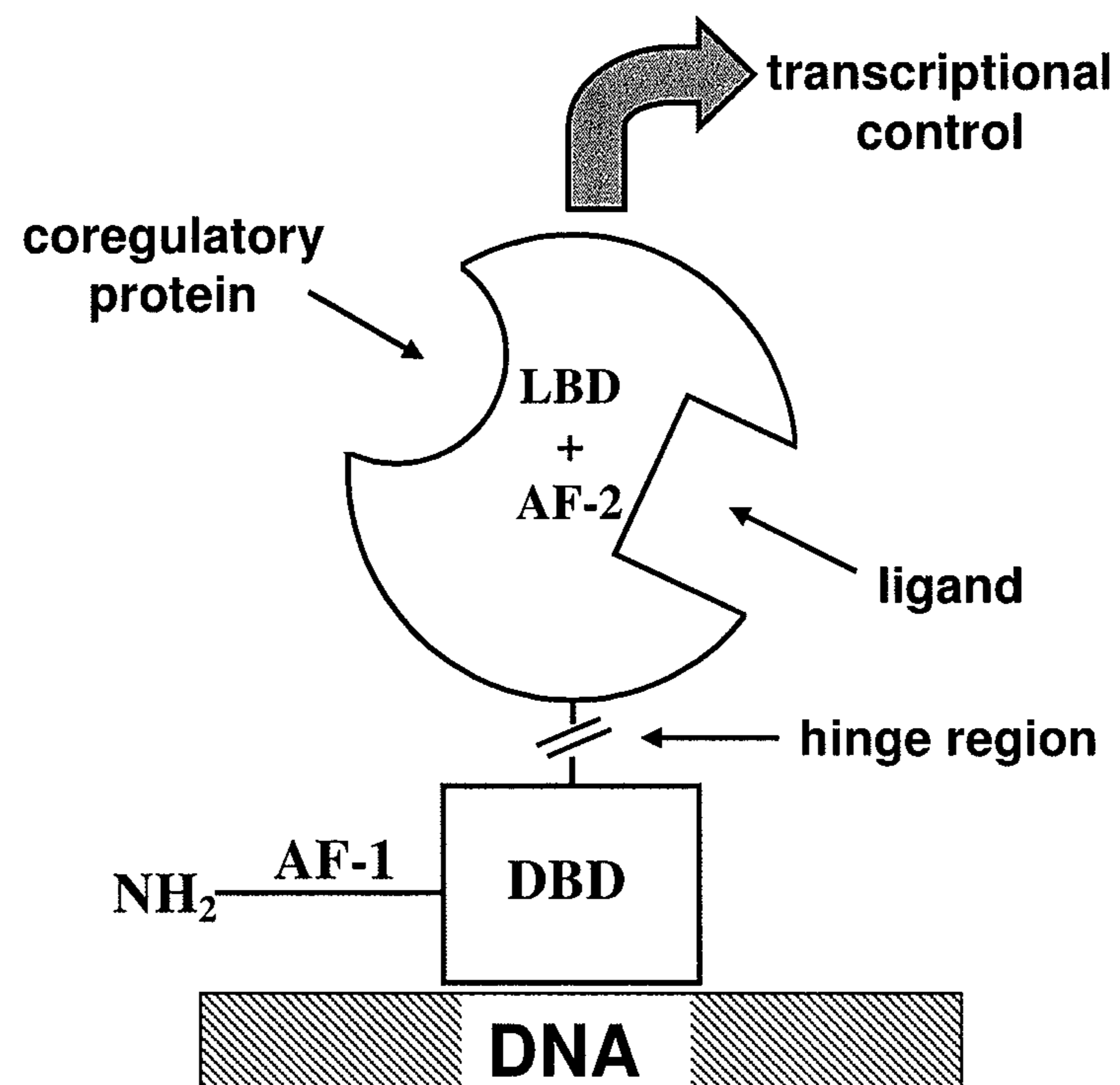


FIGURE 2

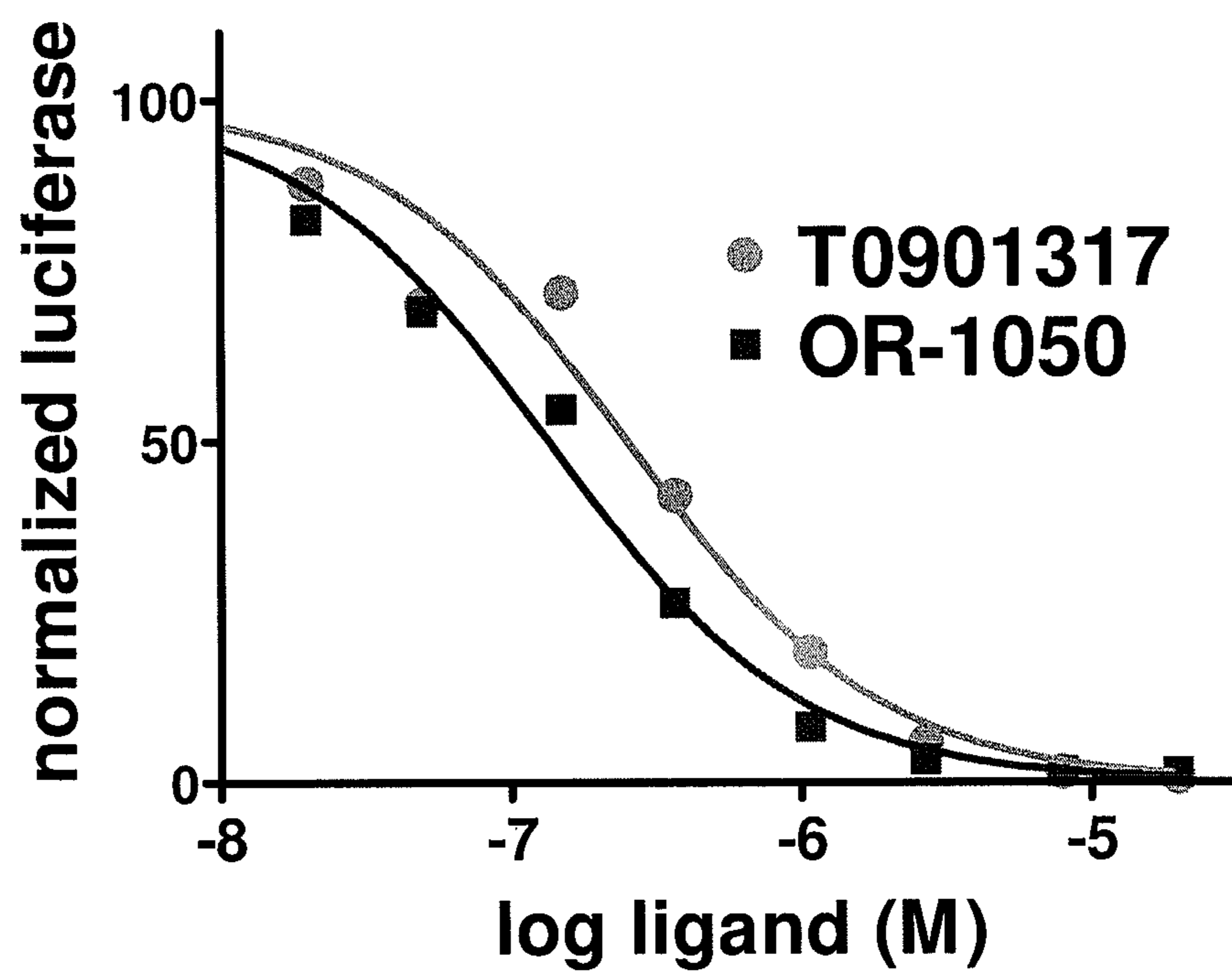


FIGURE 3.

FIG. 4A

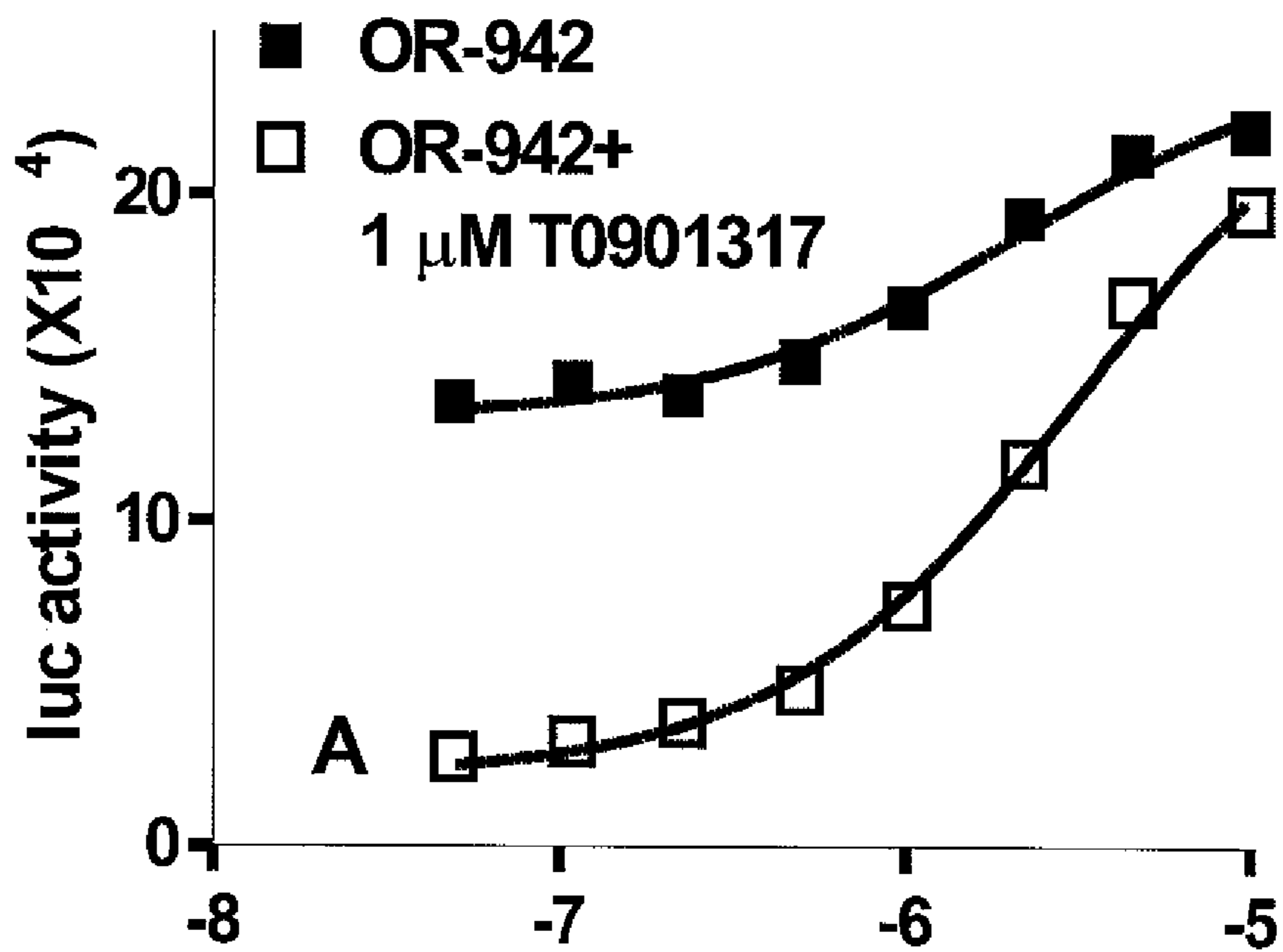
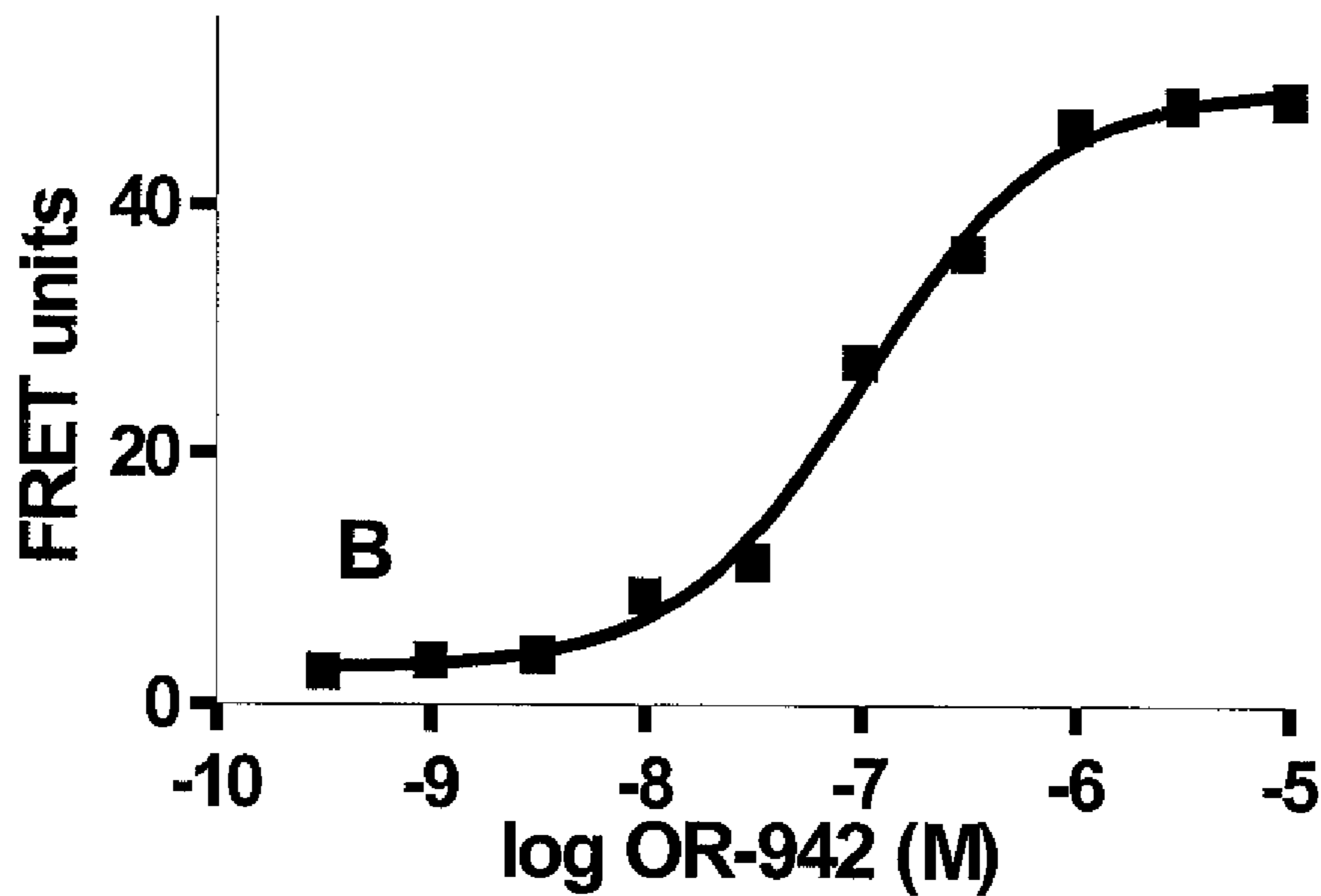


FIG. 4B



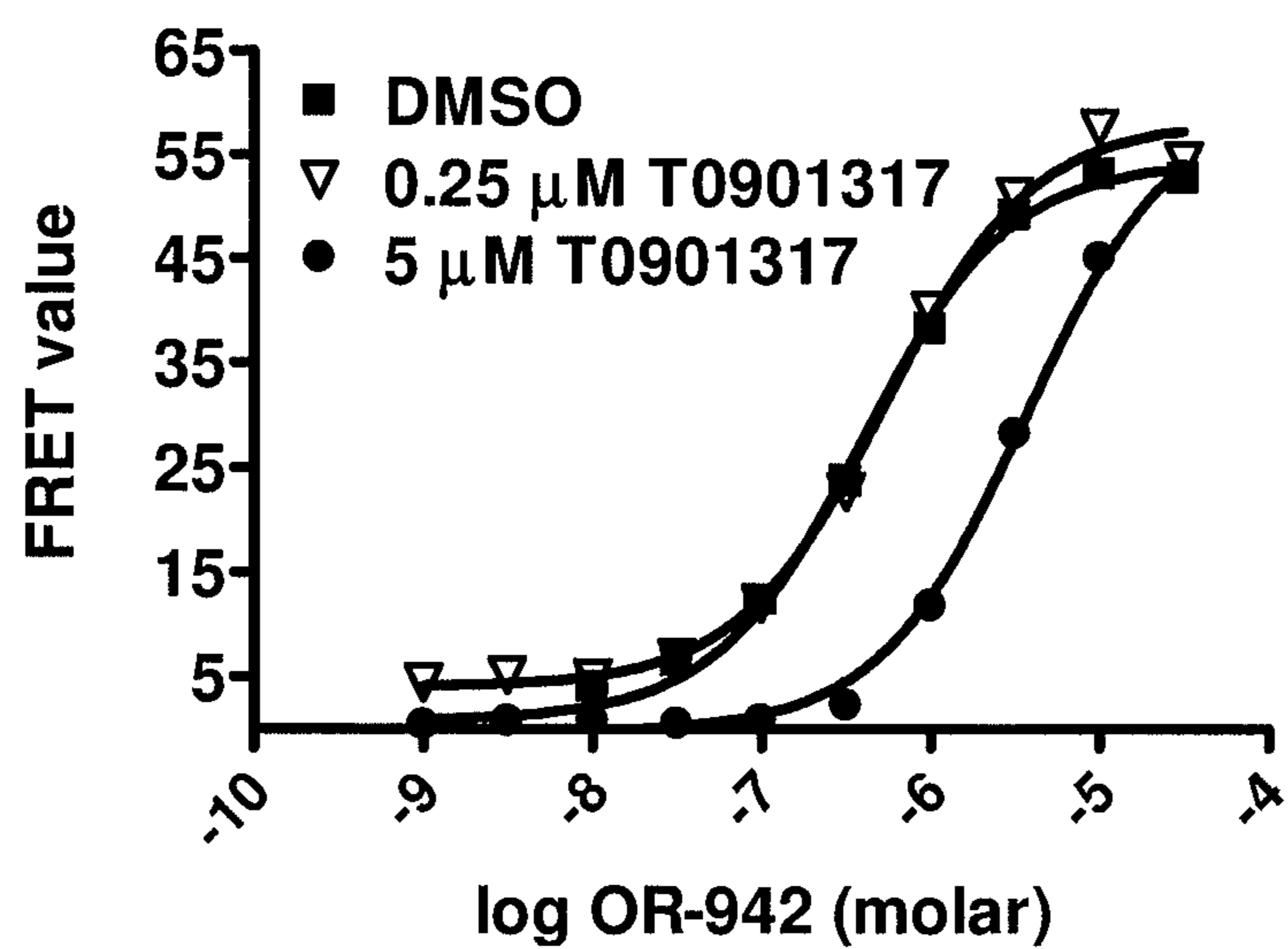


FIGURE 5.

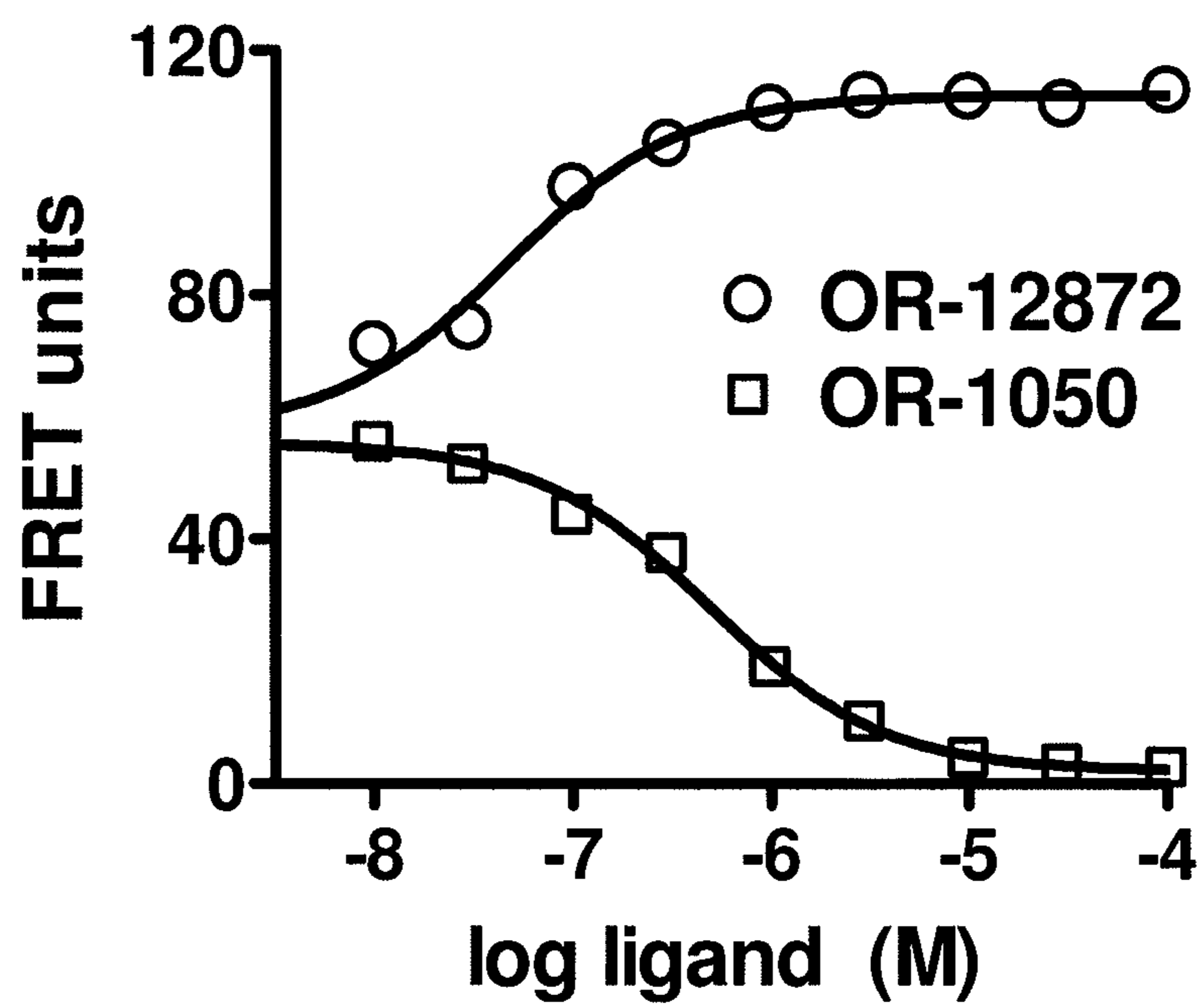


FIGURE 6.



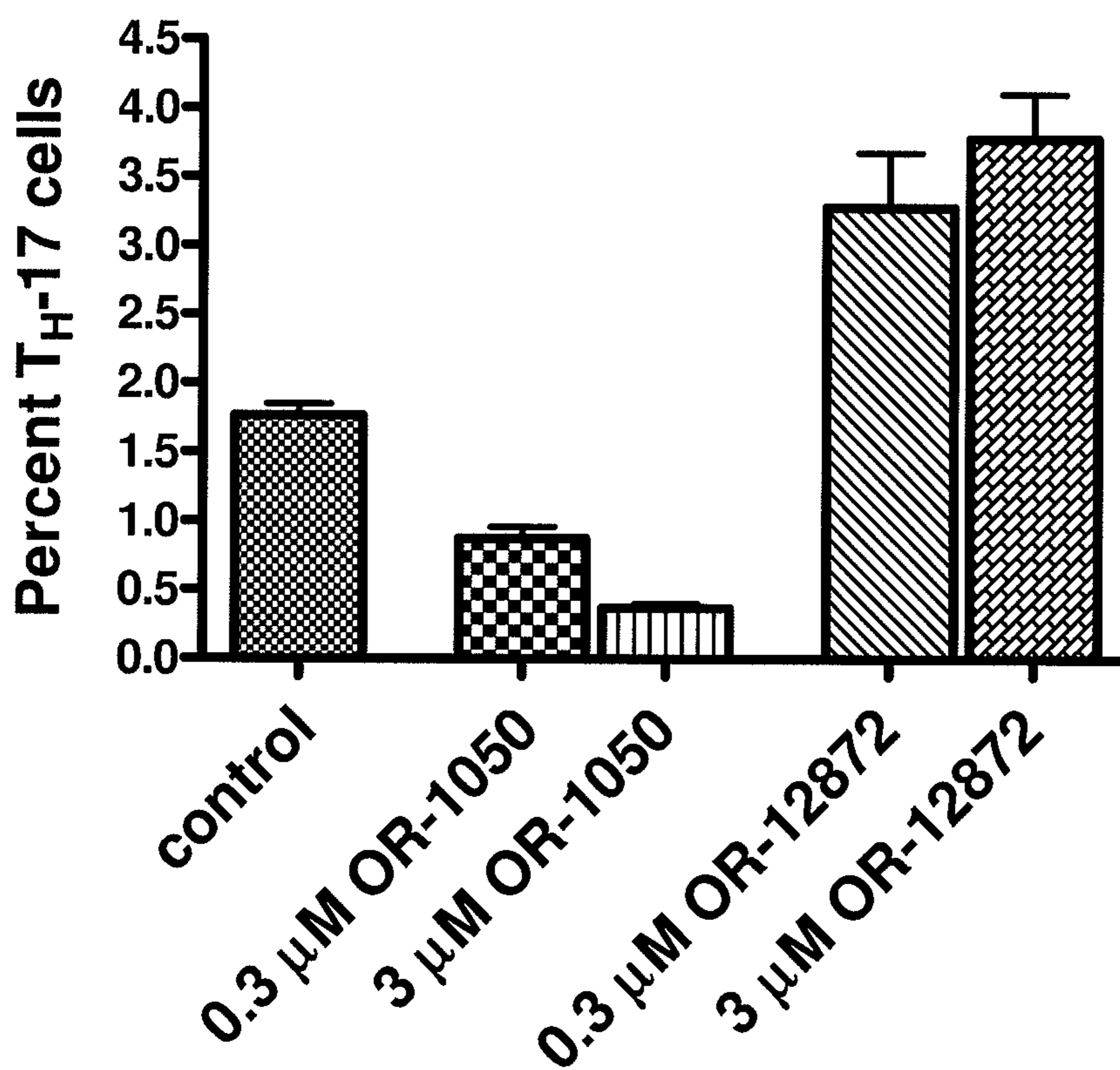


FIGURE 7.

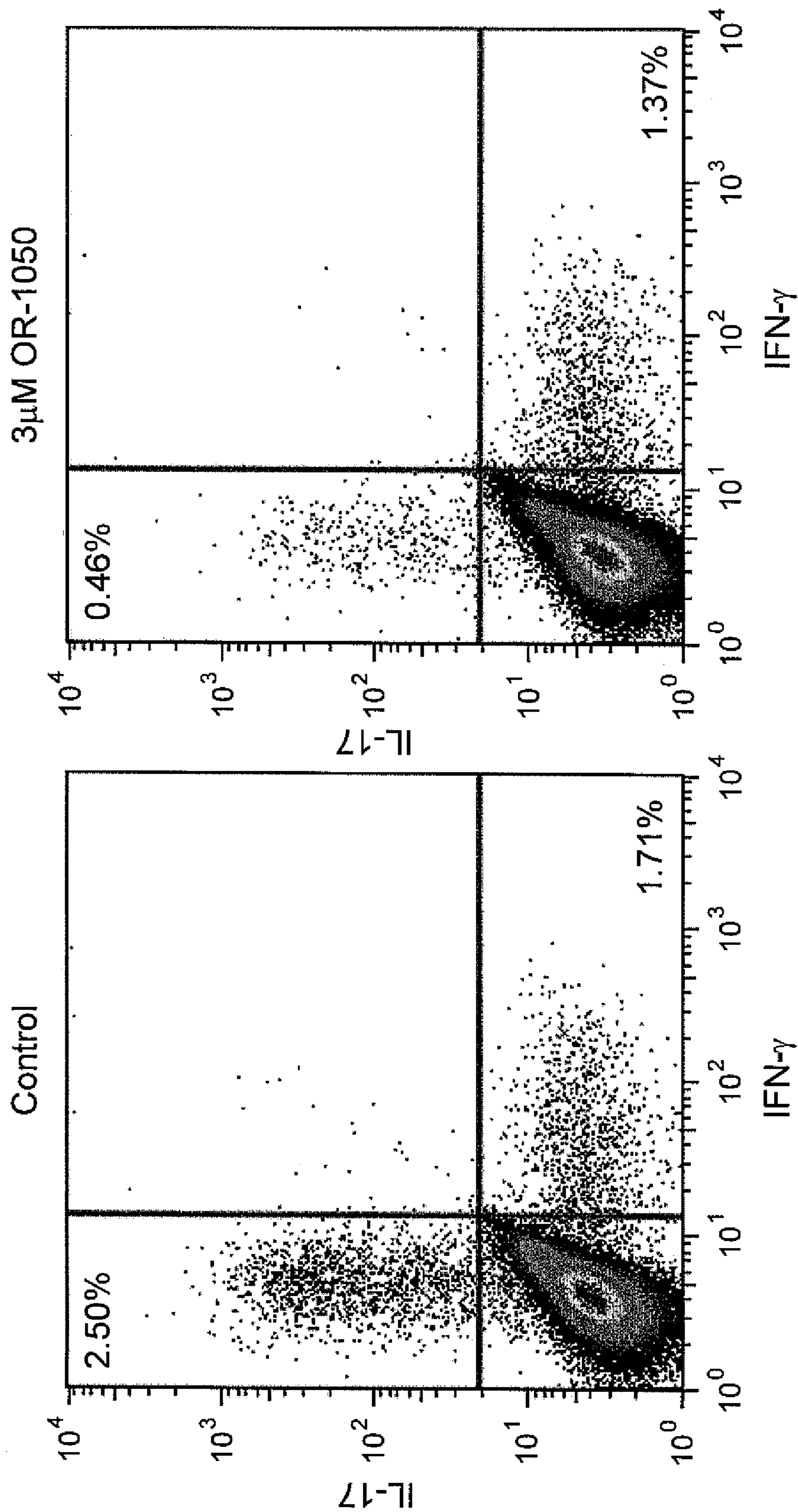


FIG. 8A

FIG. 8B

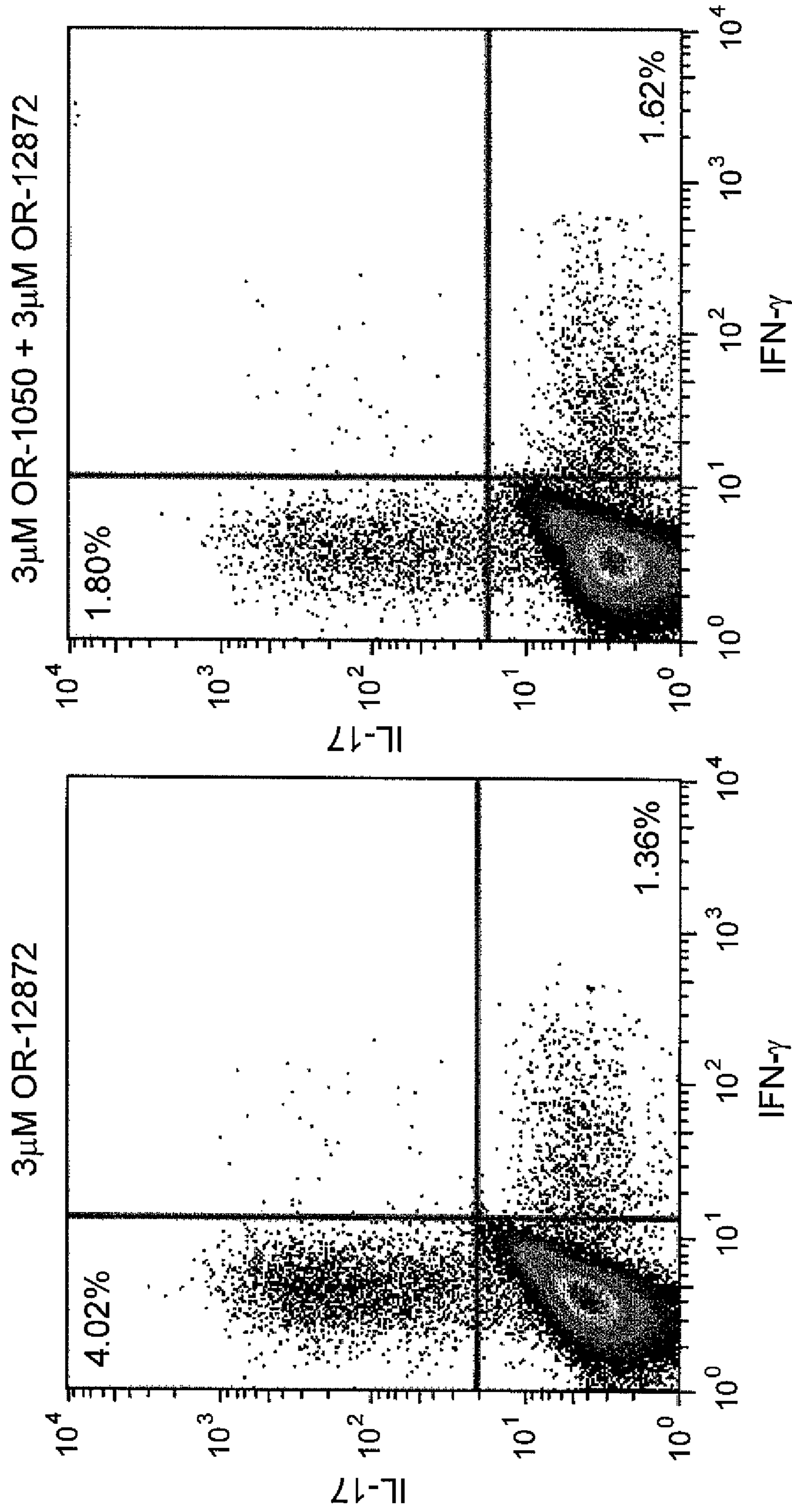


FIG. 8C

FIG. 8D

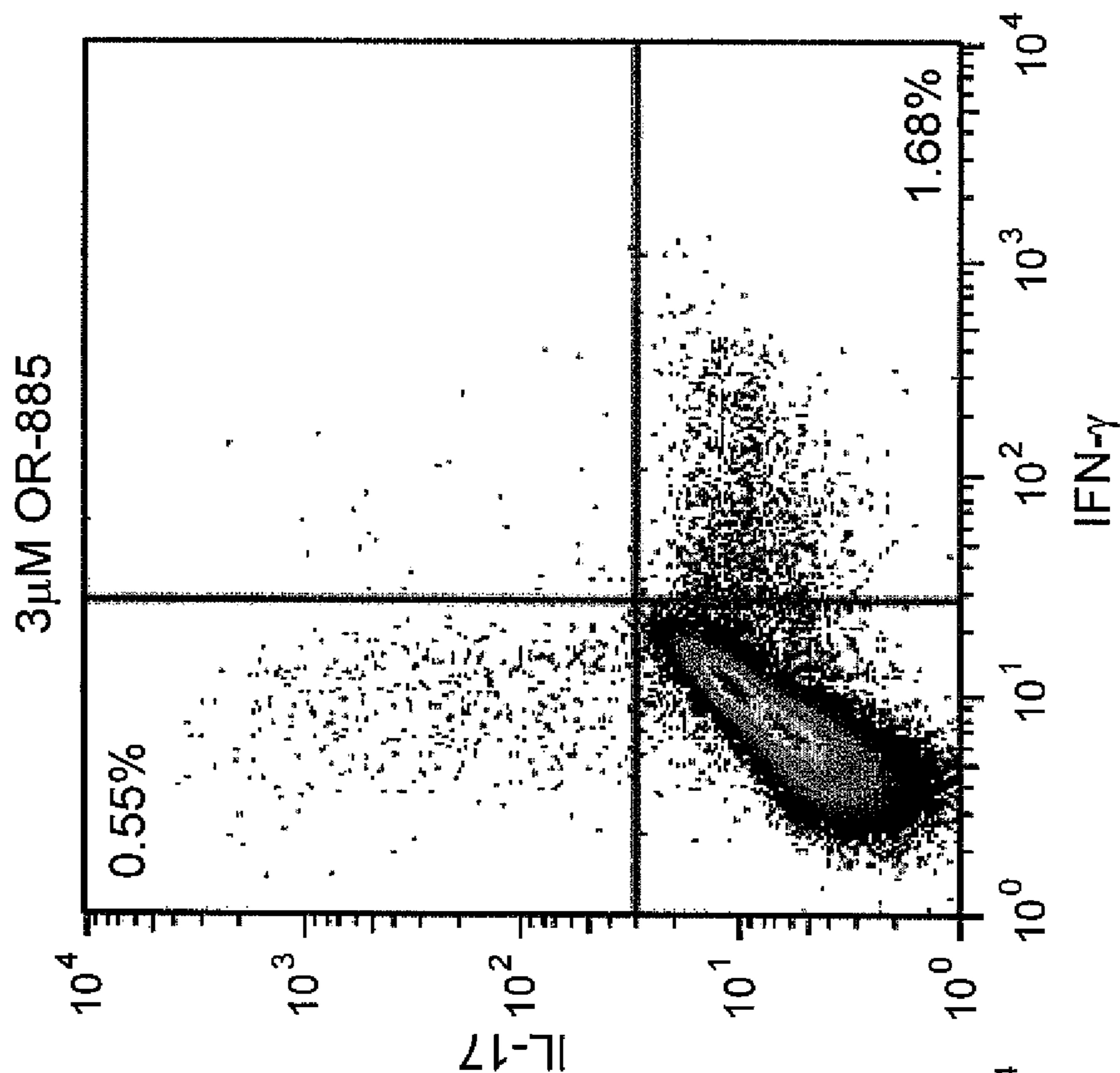


FIG. 9B

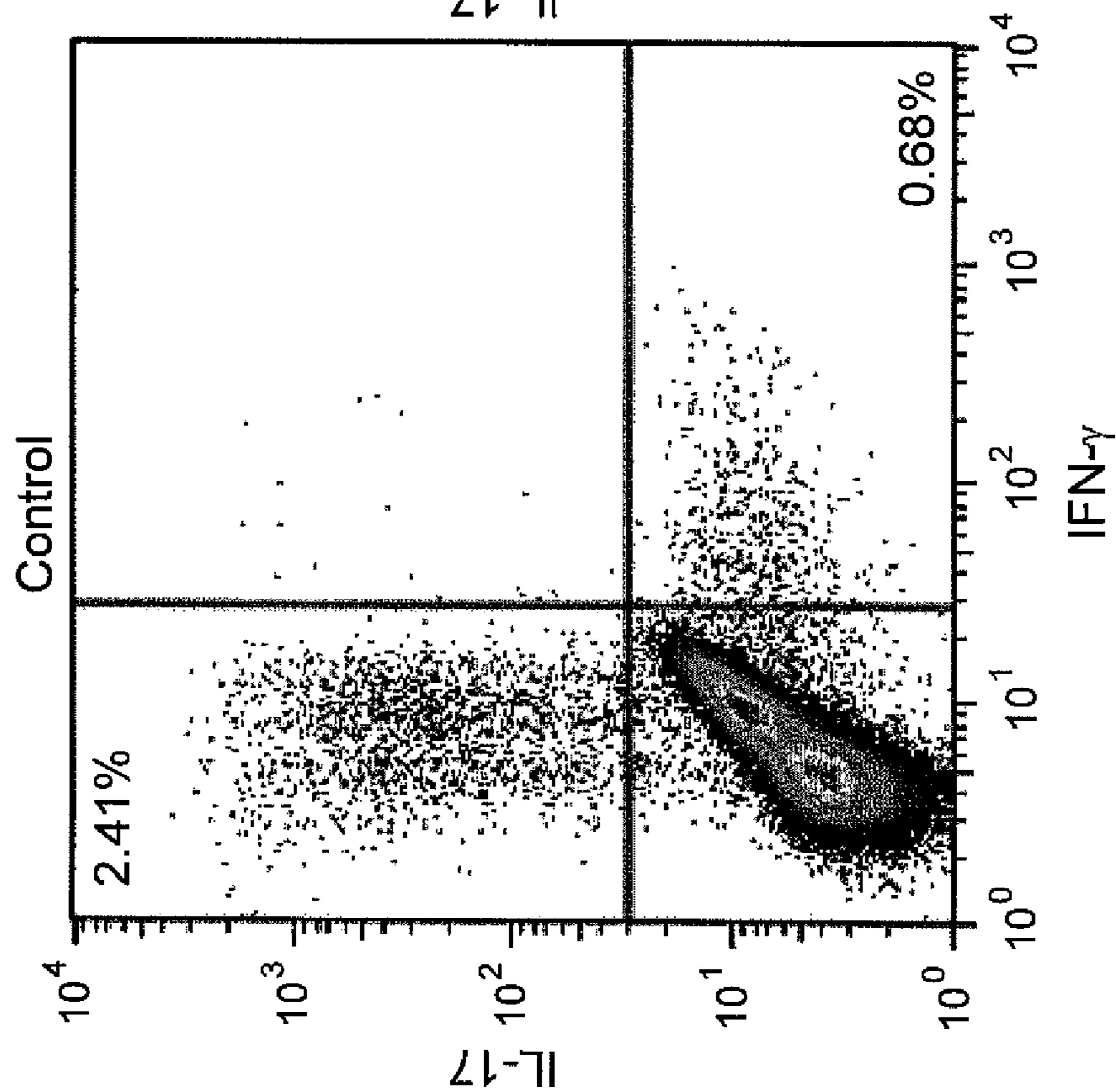


FIG. 9A

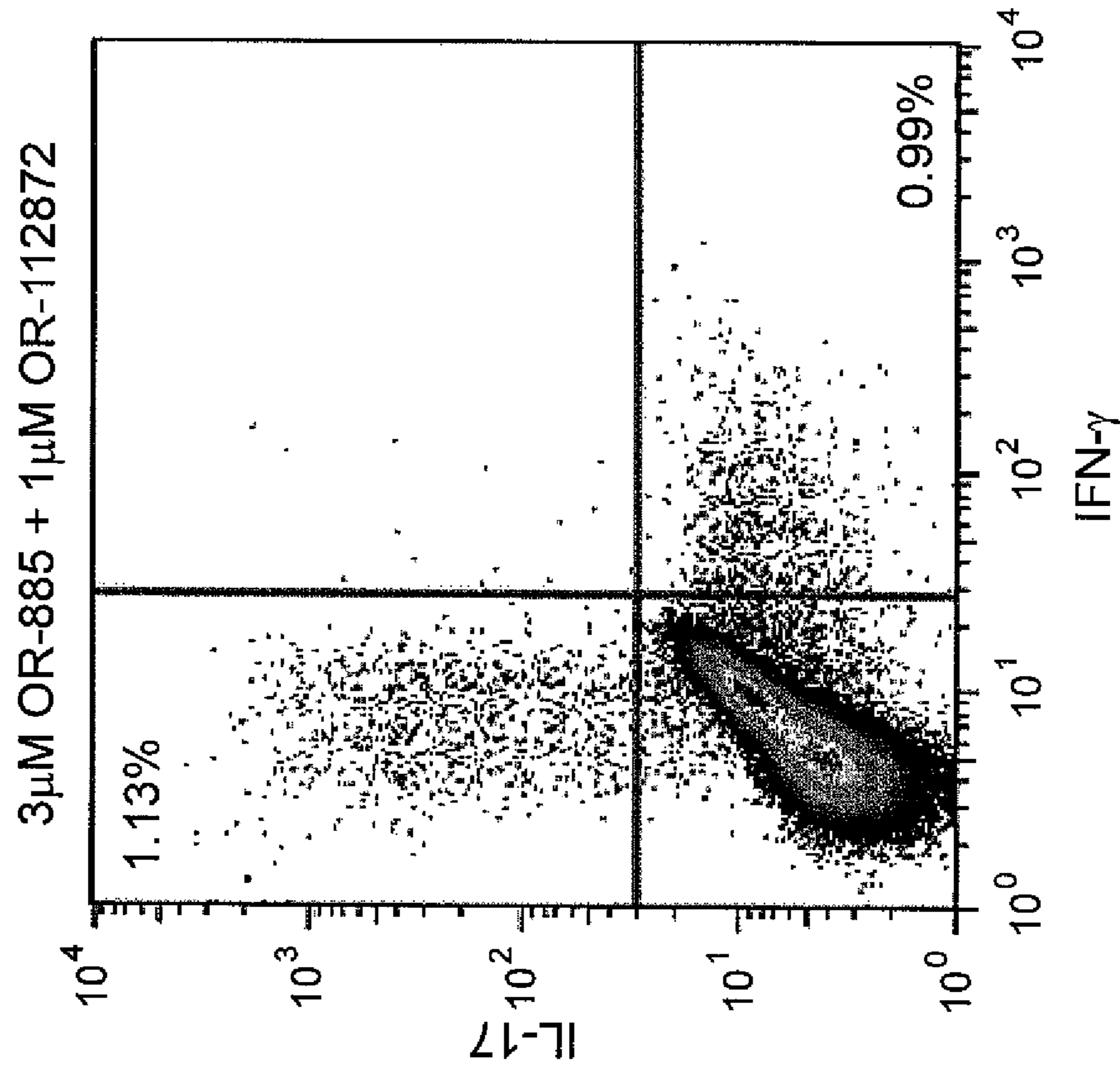


FIG. 9D

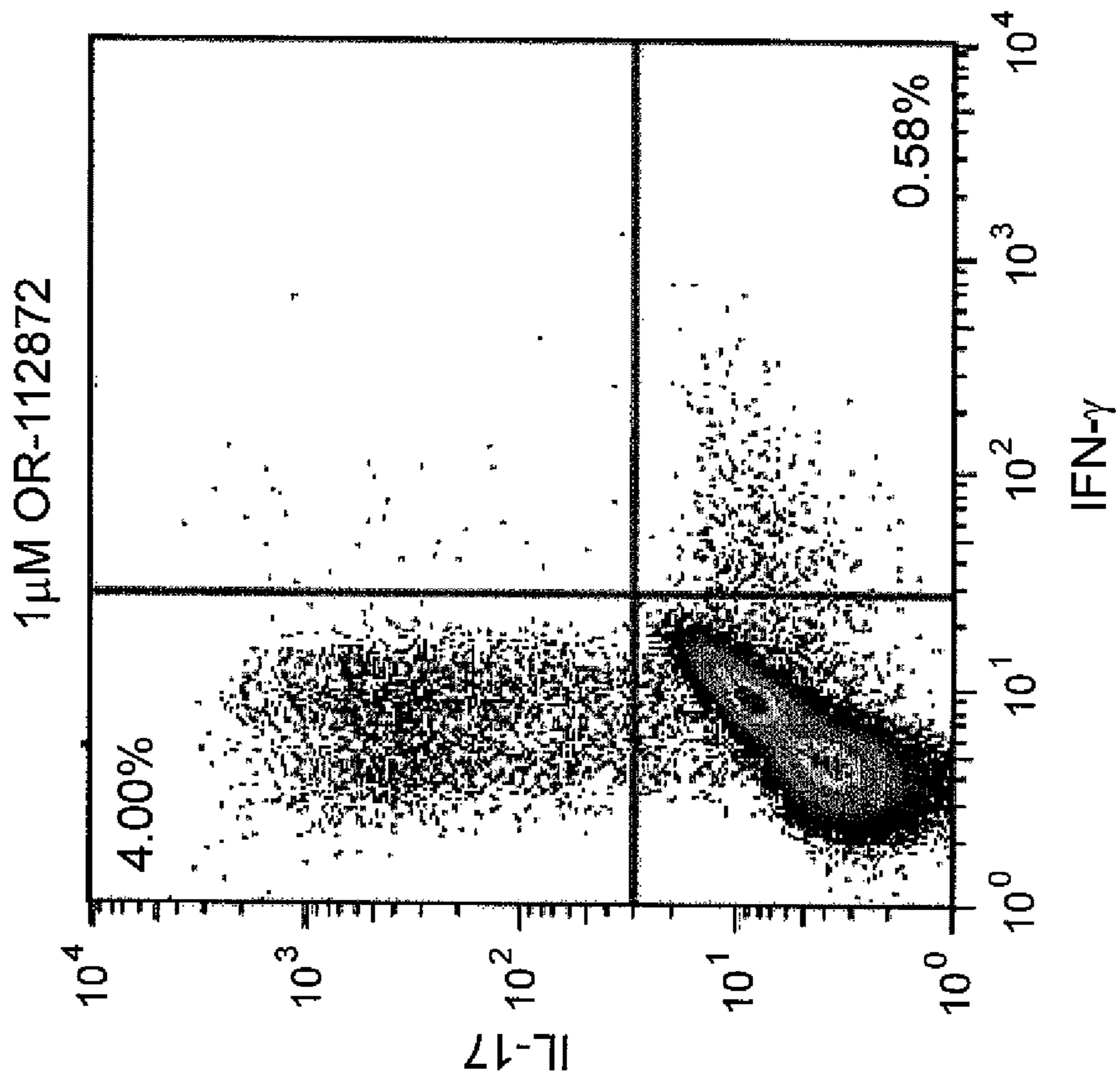


FIG. 9C

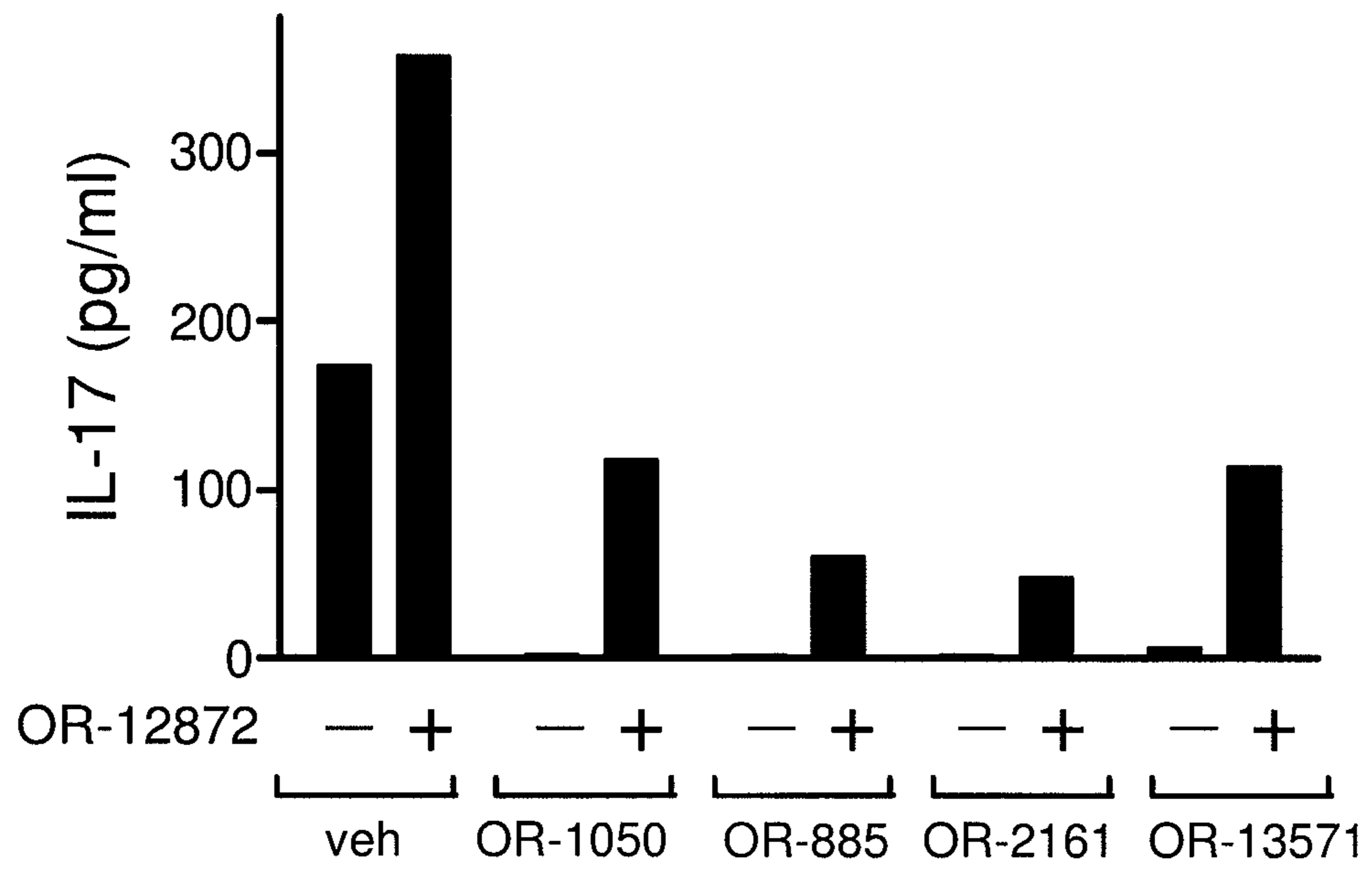


FIGURE 10.

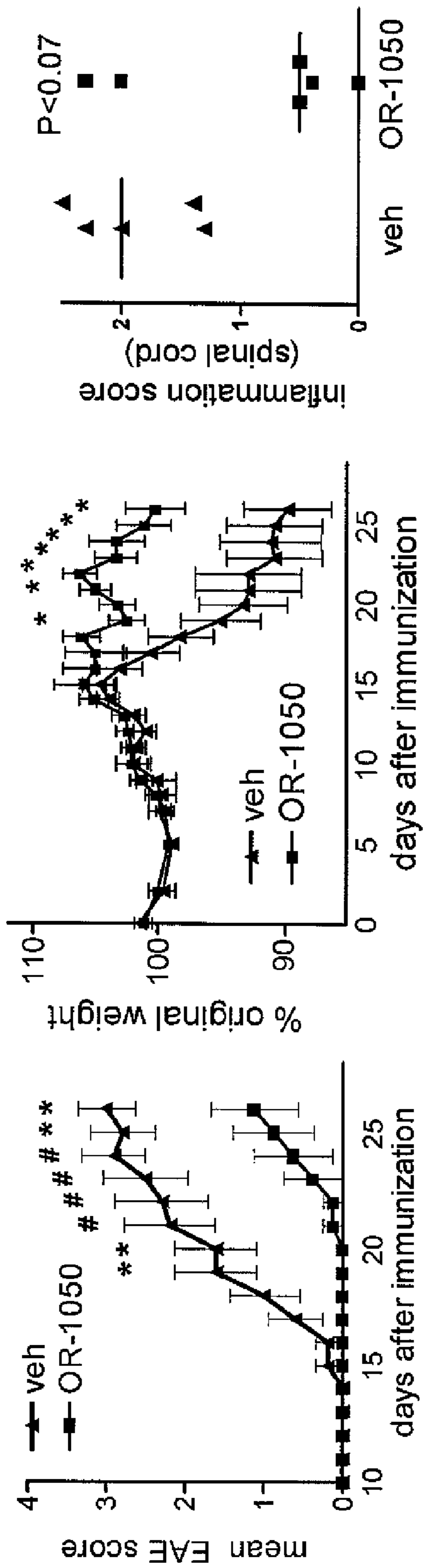


FIG. 11A

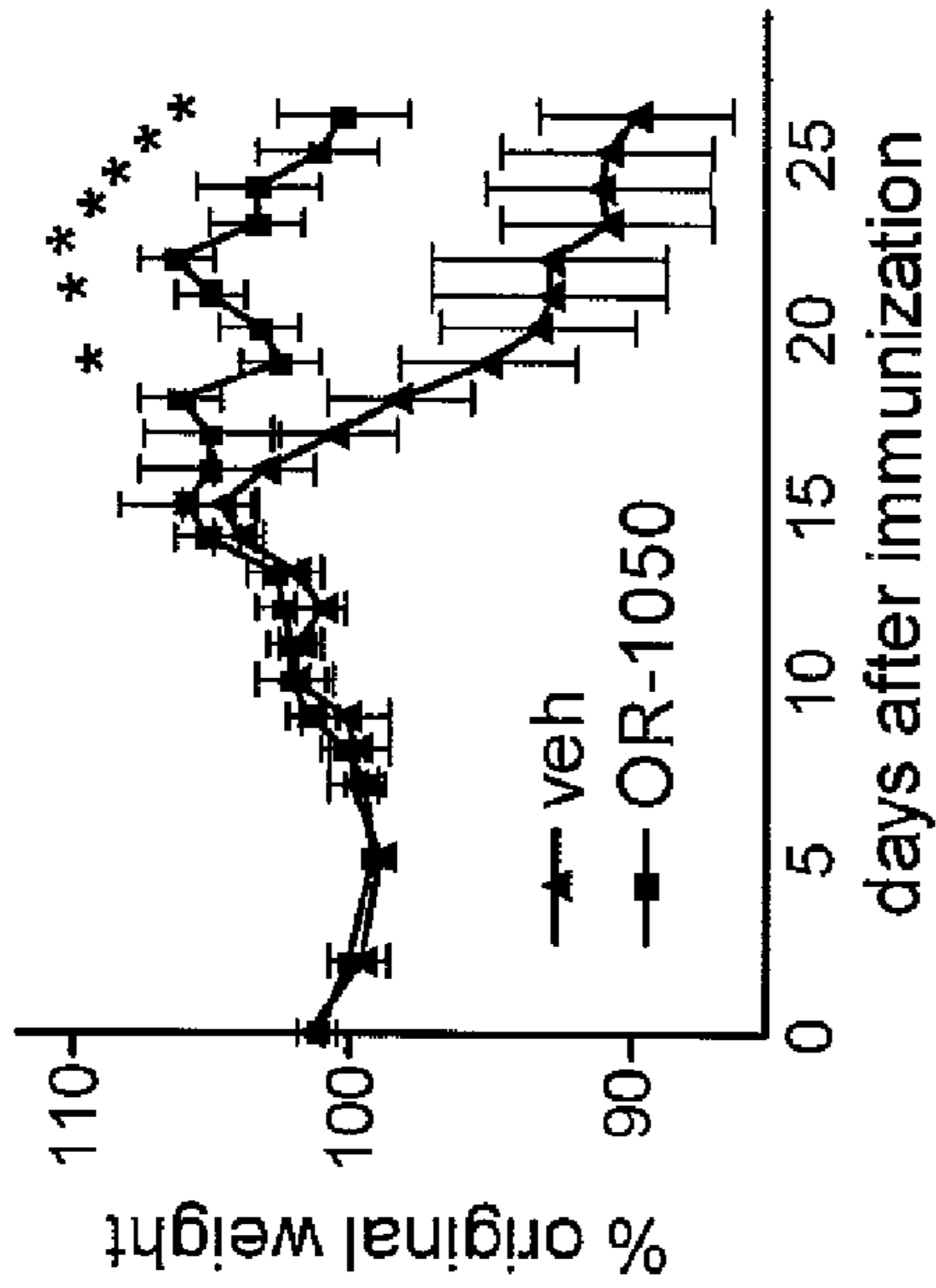


FIG. 11B

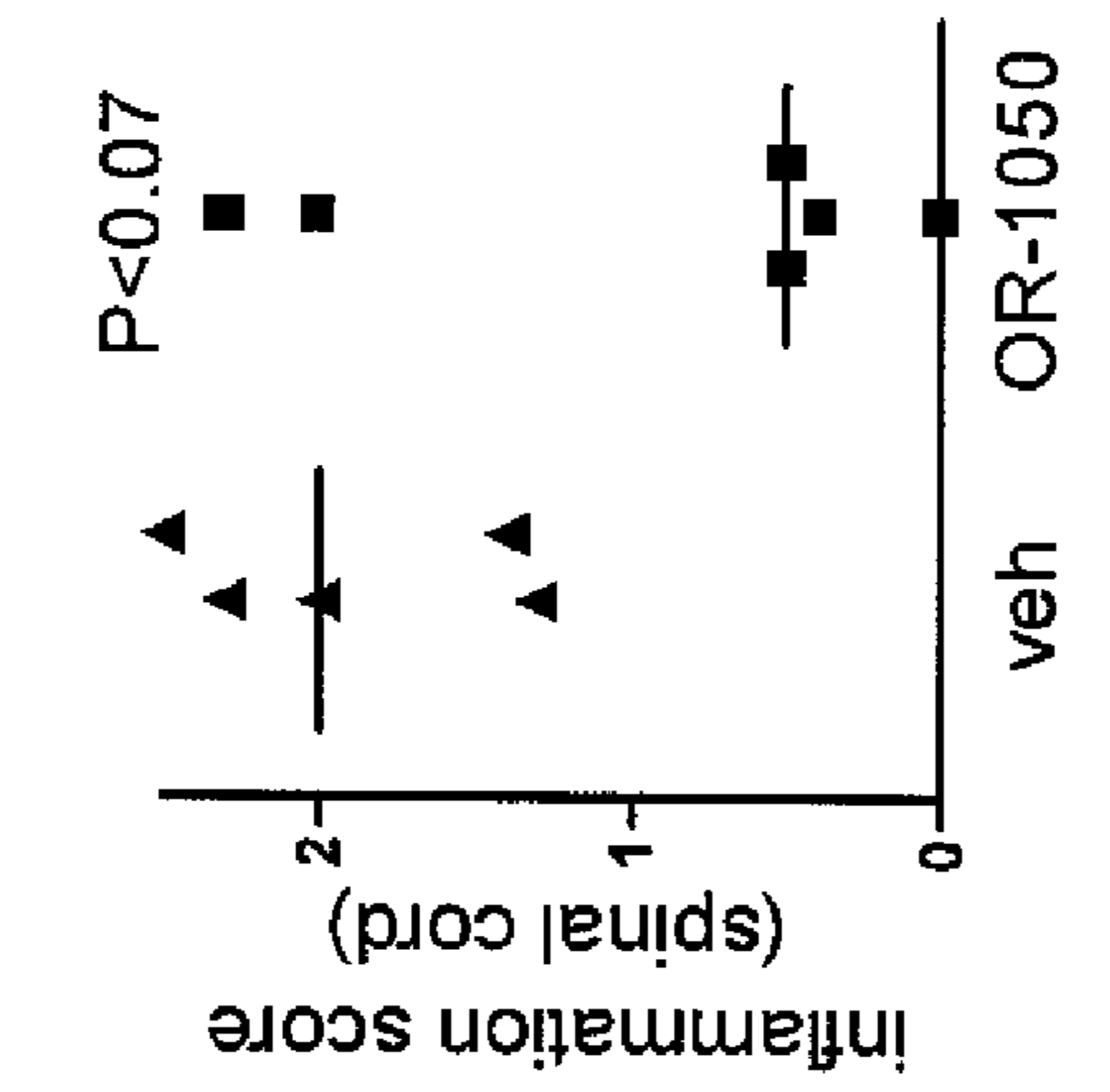


FIG. 11C

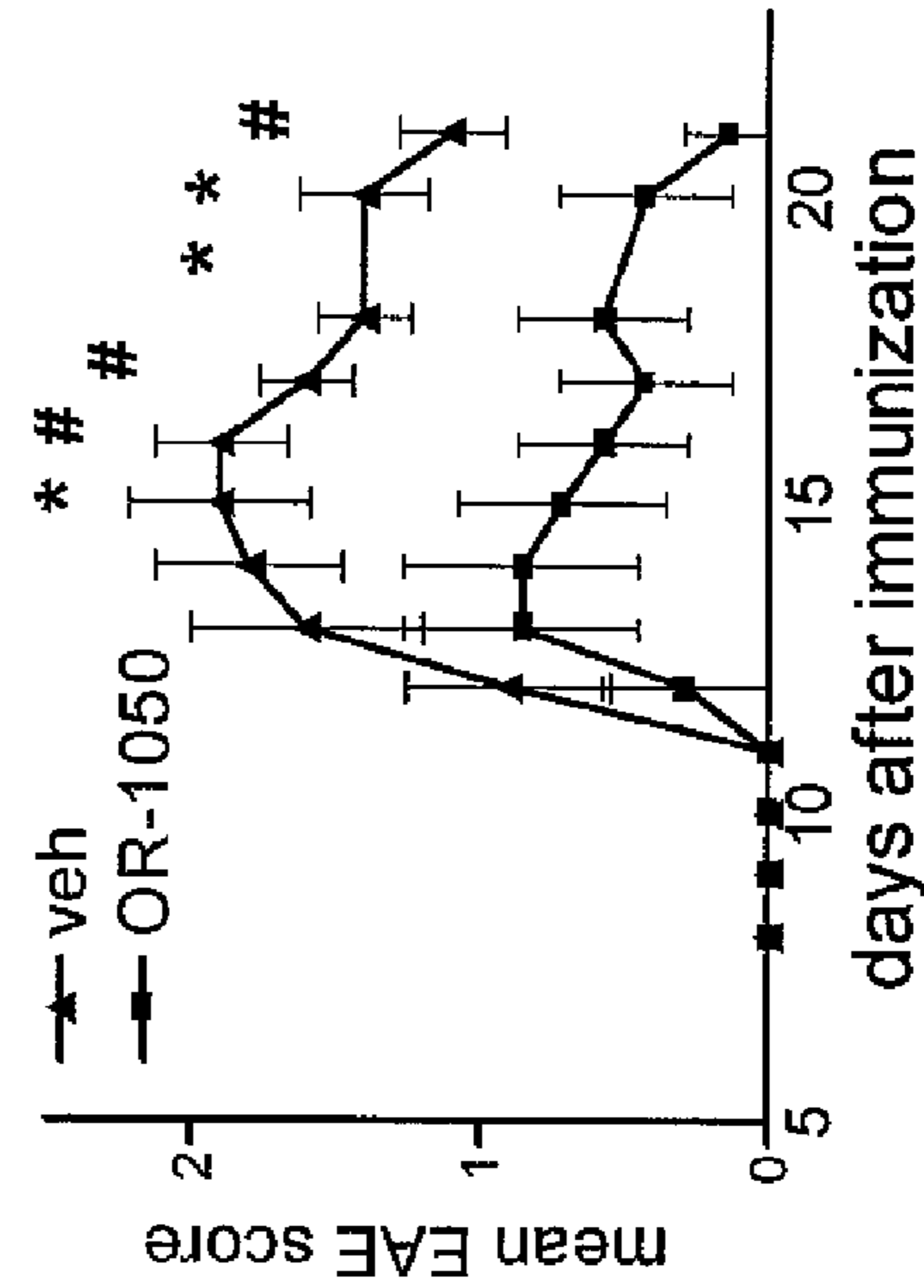


FIG. 11D

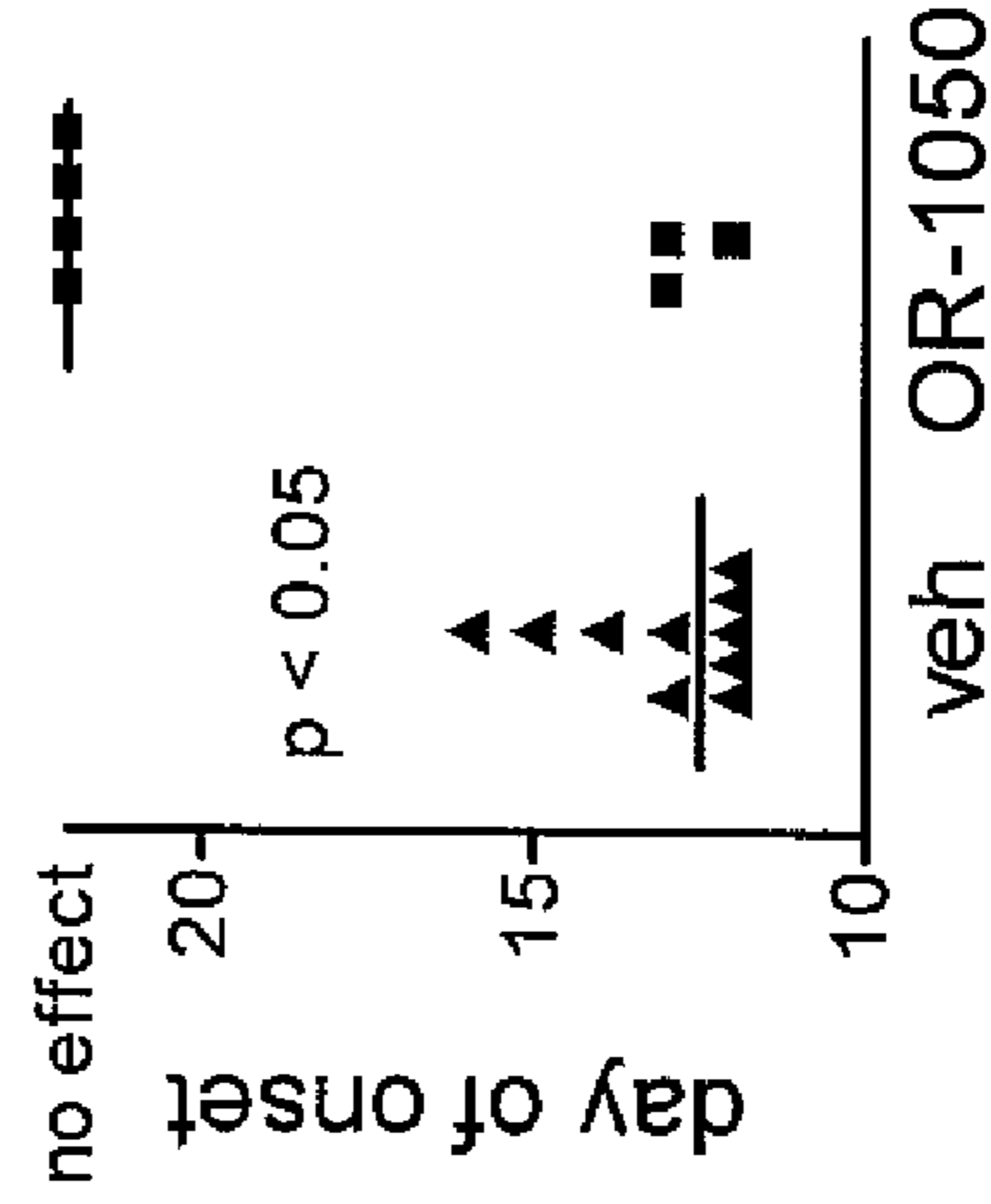


FIG. 11E

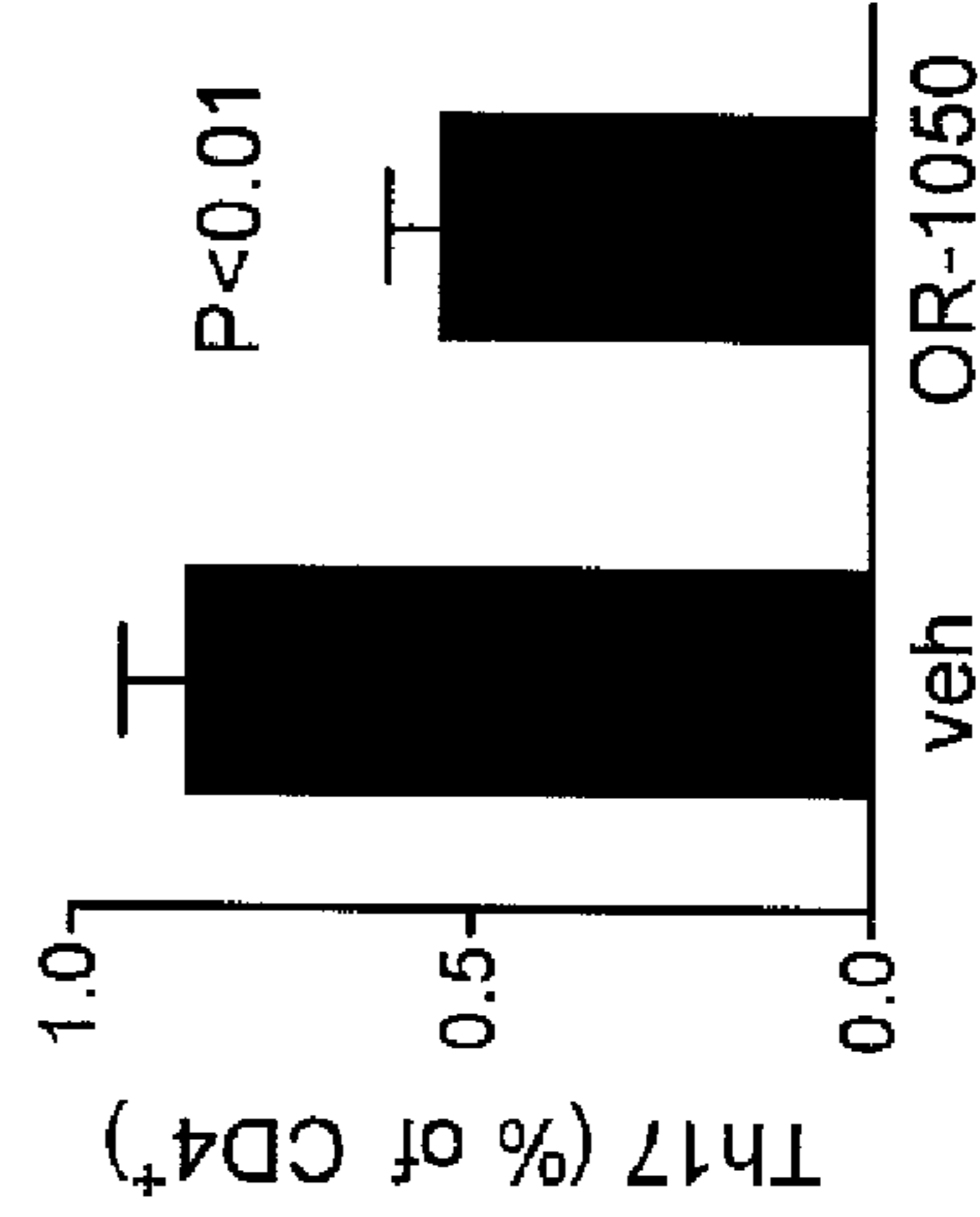


FIG. 11F

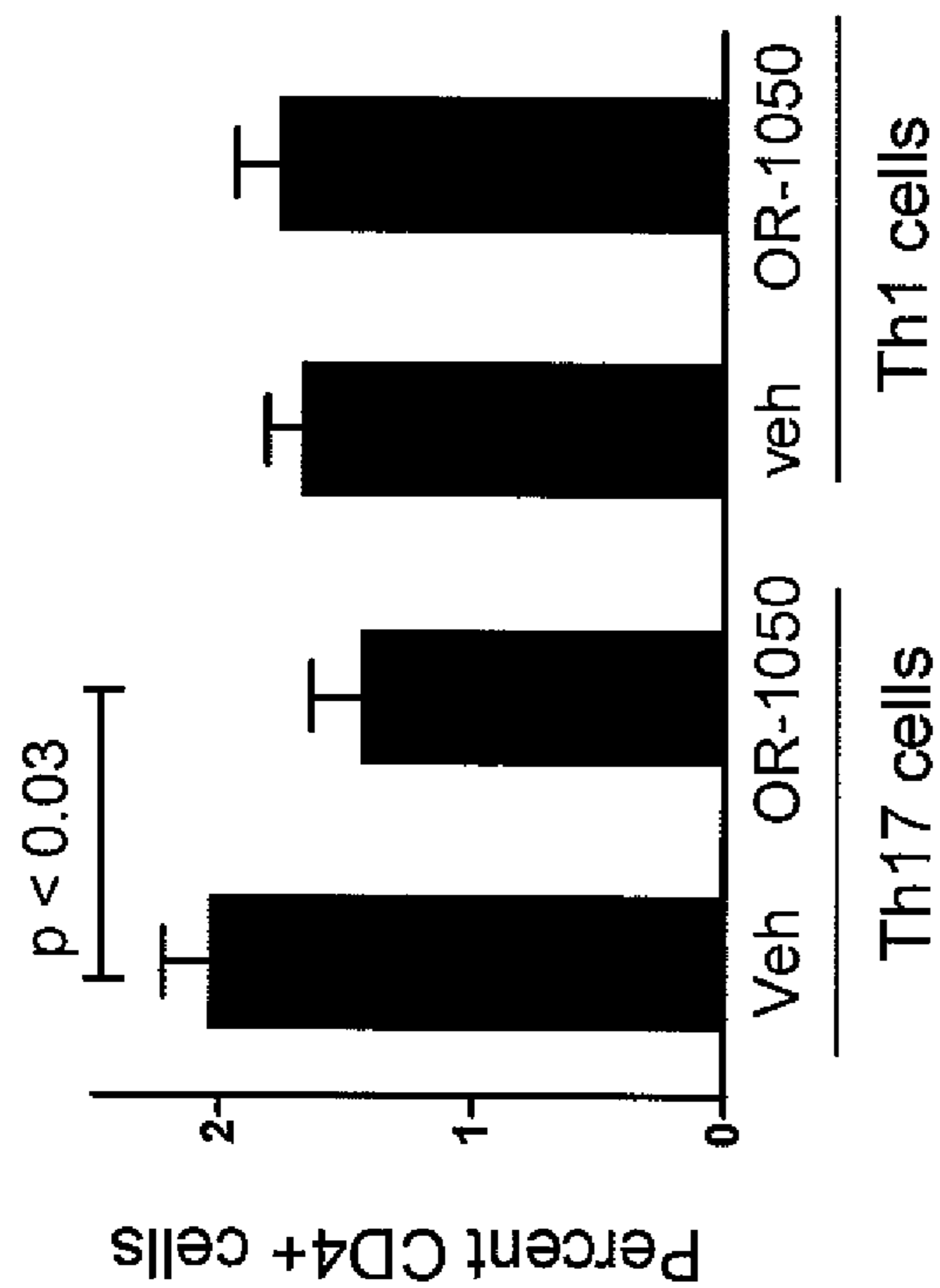


FIG. 11G



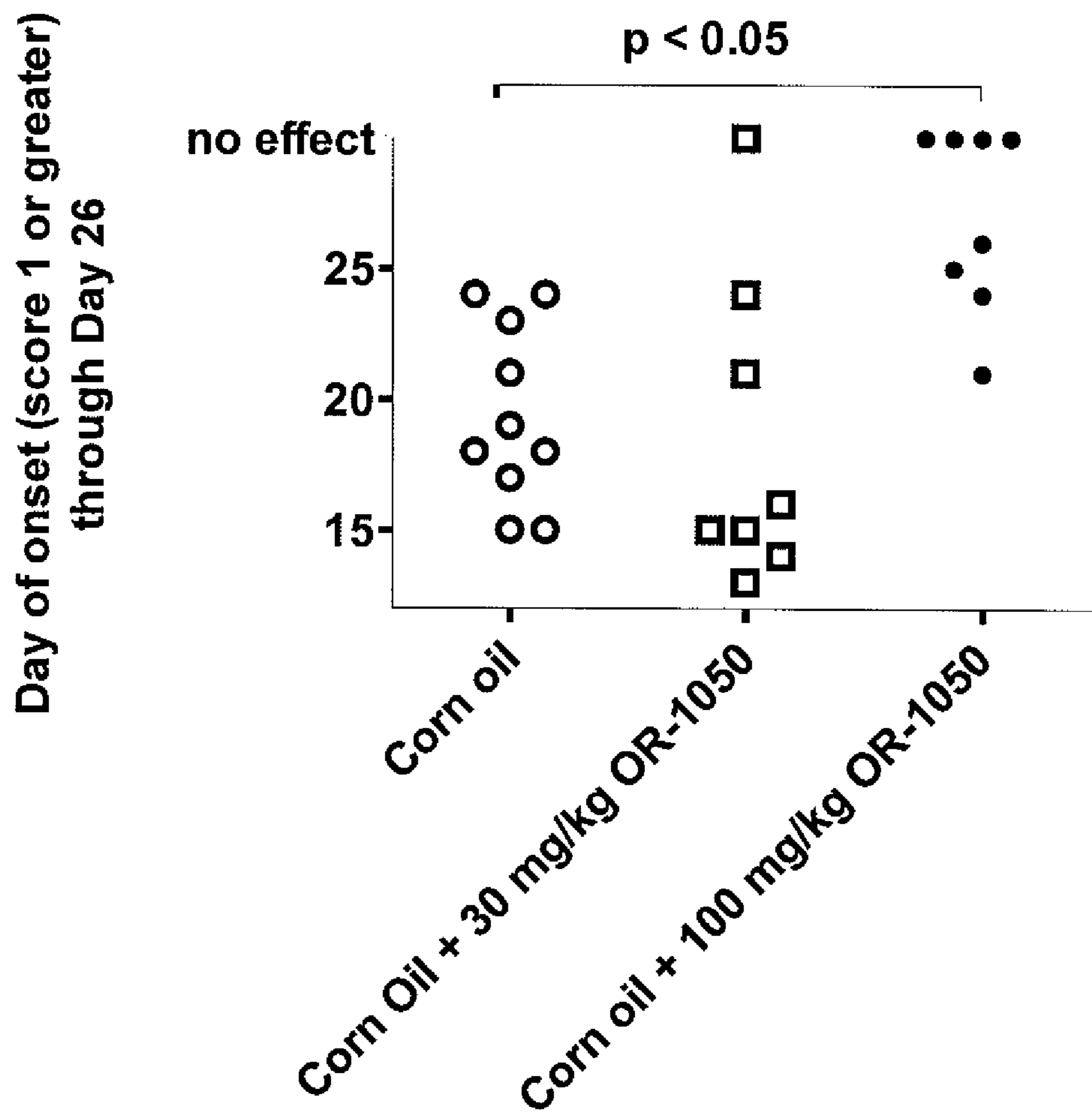


FIG. 12A

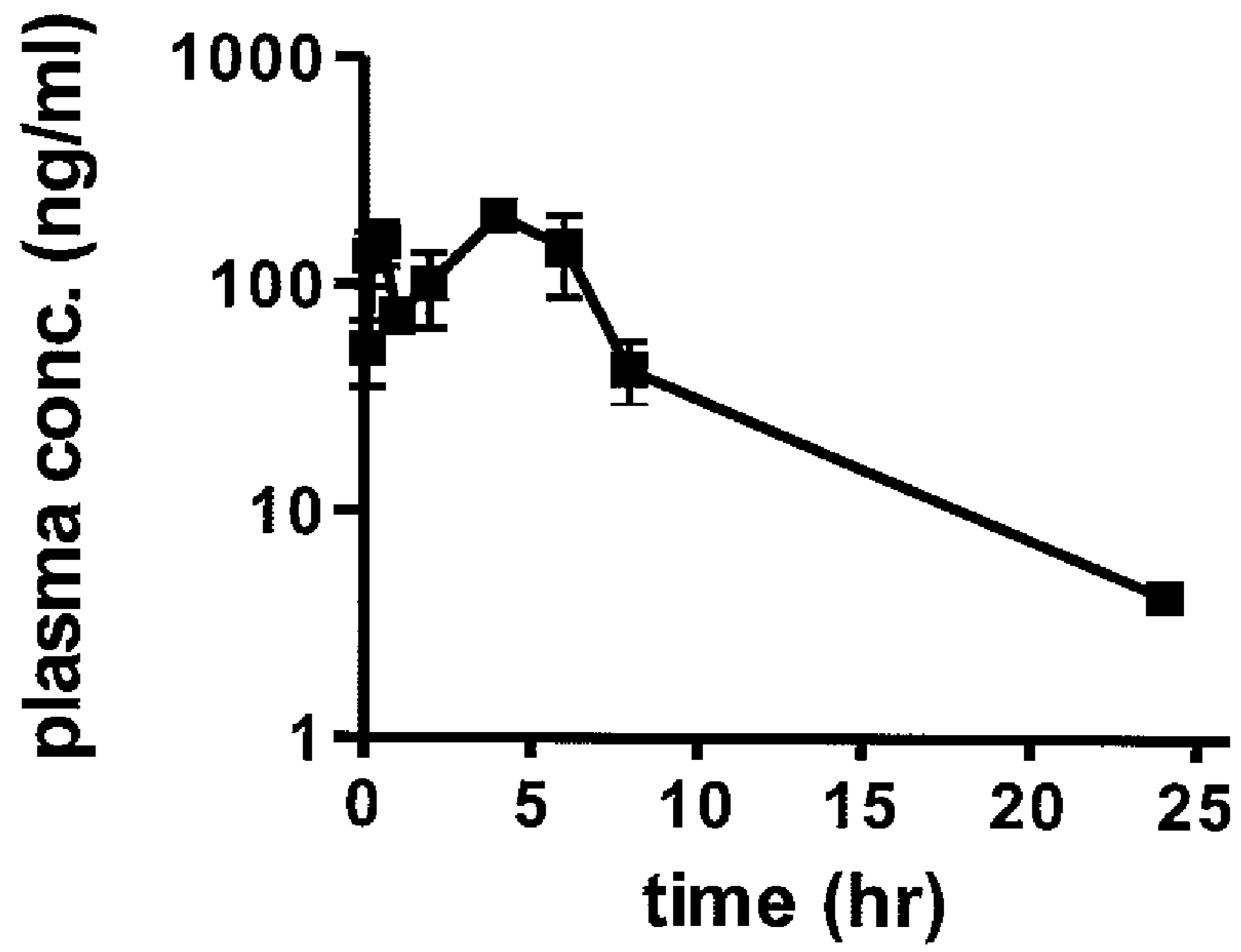


FIG. 12B

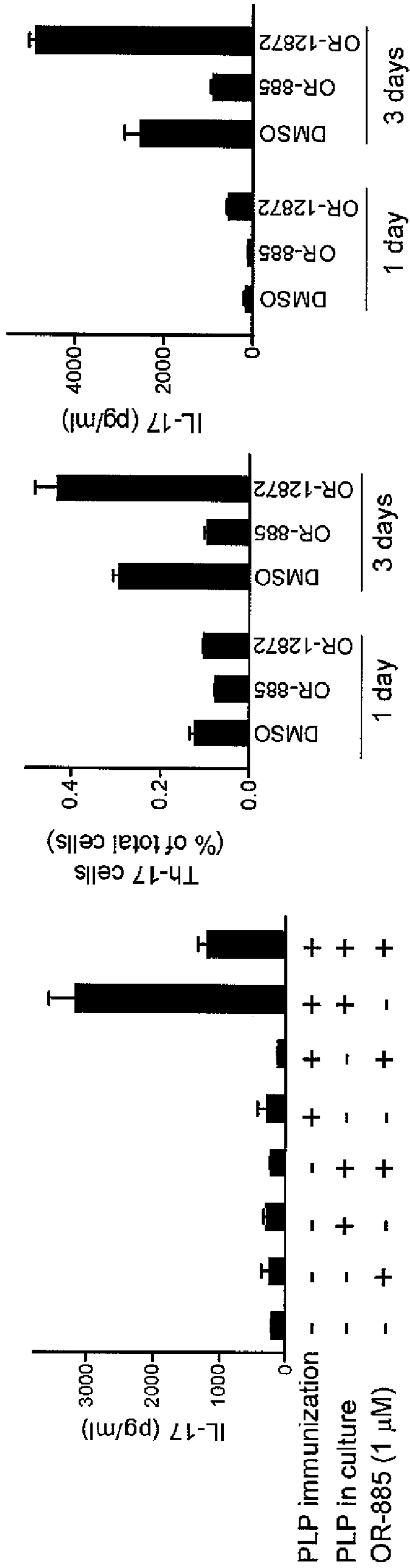


FIG. 13A

FIG. 13B

FIG. 13C

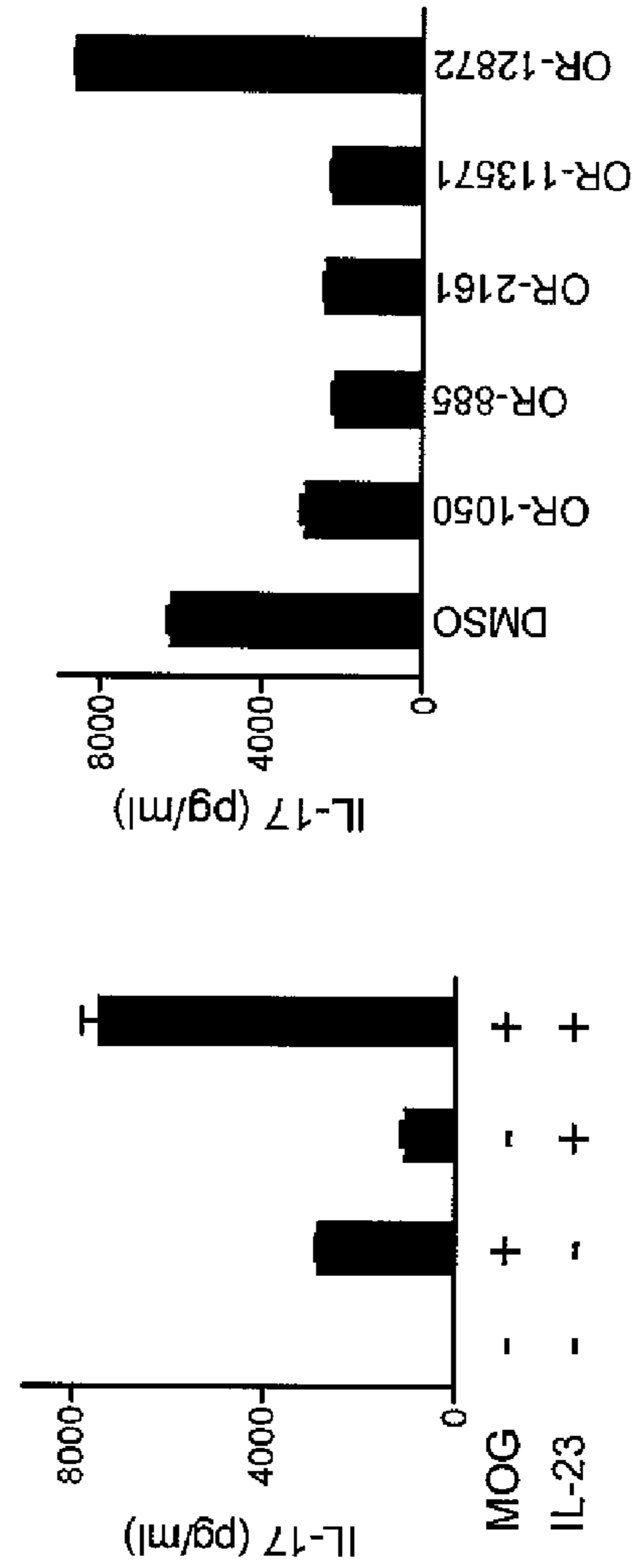


FIG. 13D

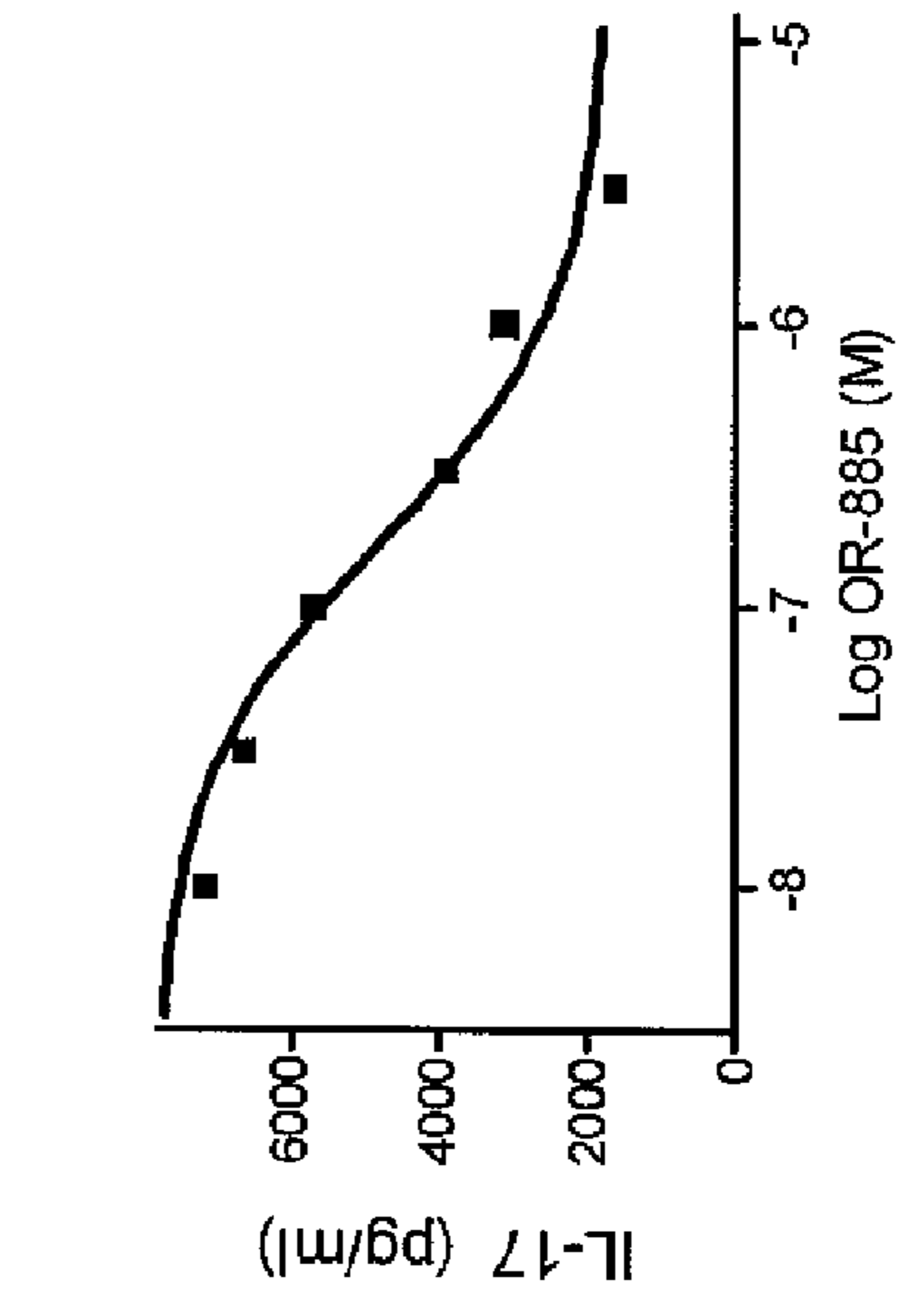


FIG. 13E

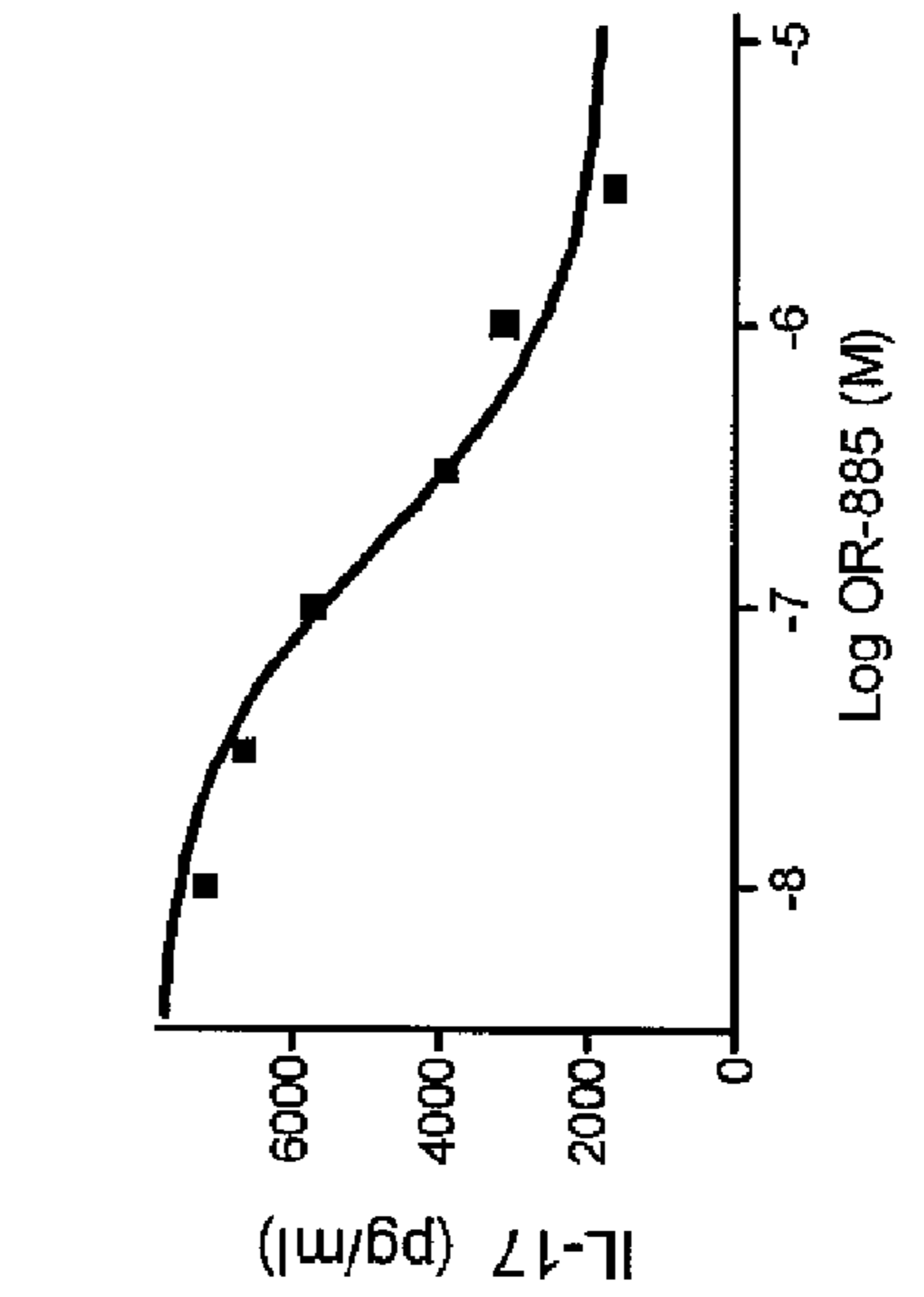


FIG. 13F

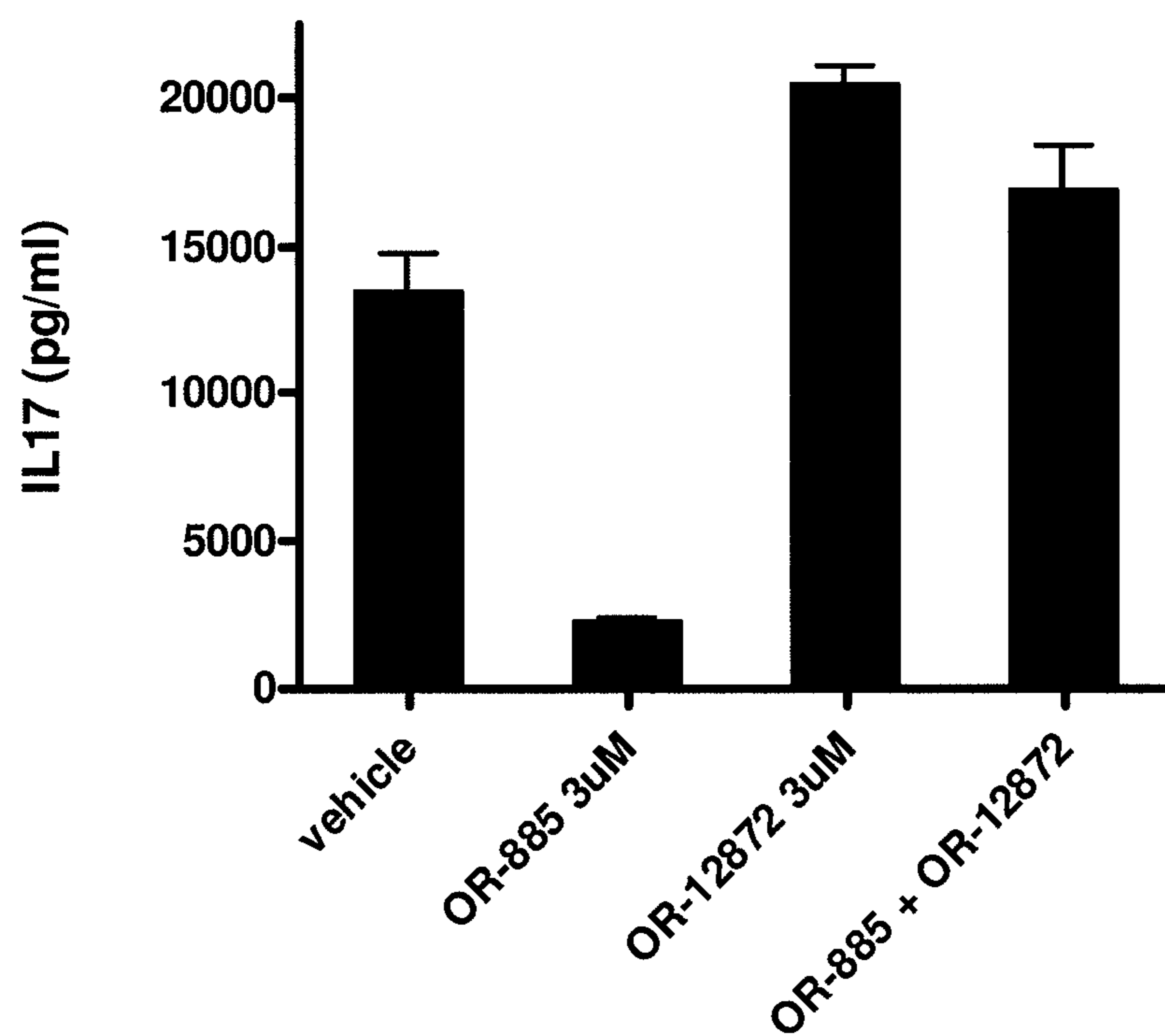


Figure 14

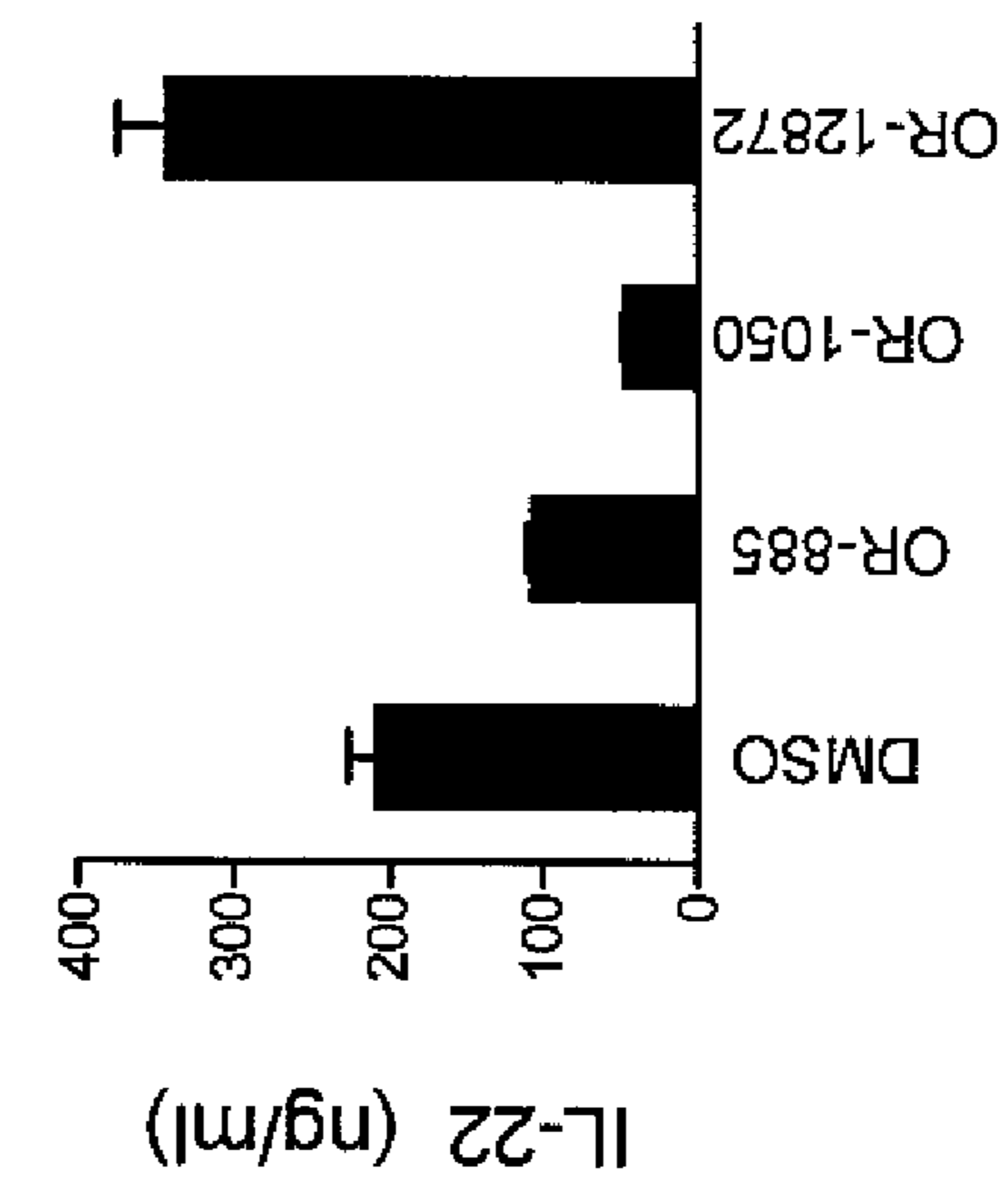


FIG. 15B

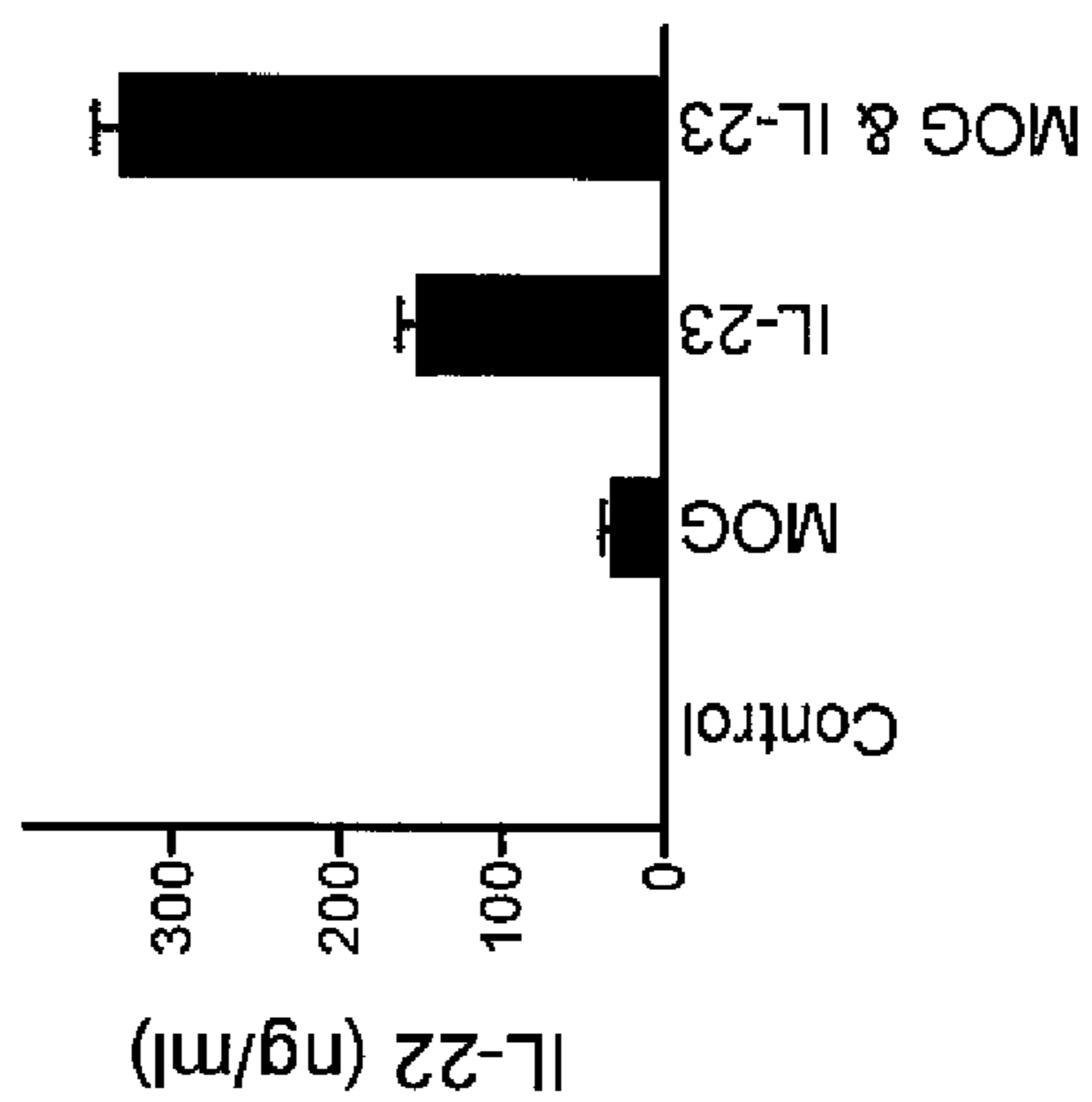
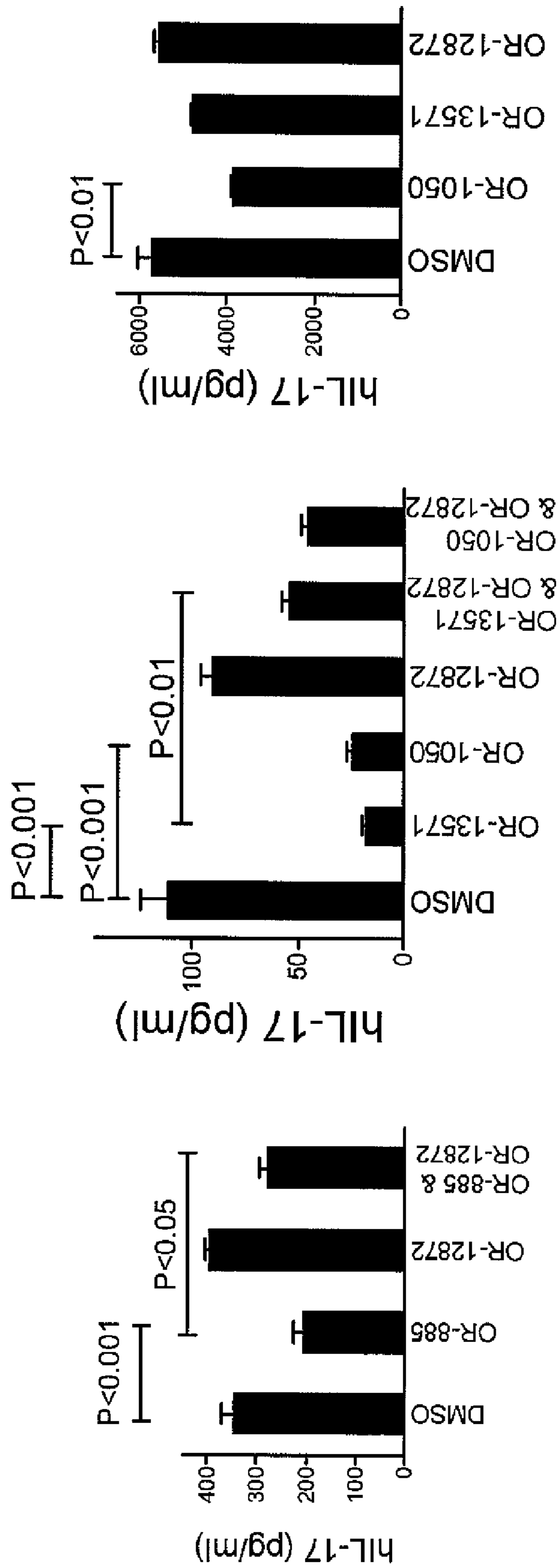


FIG. 15A



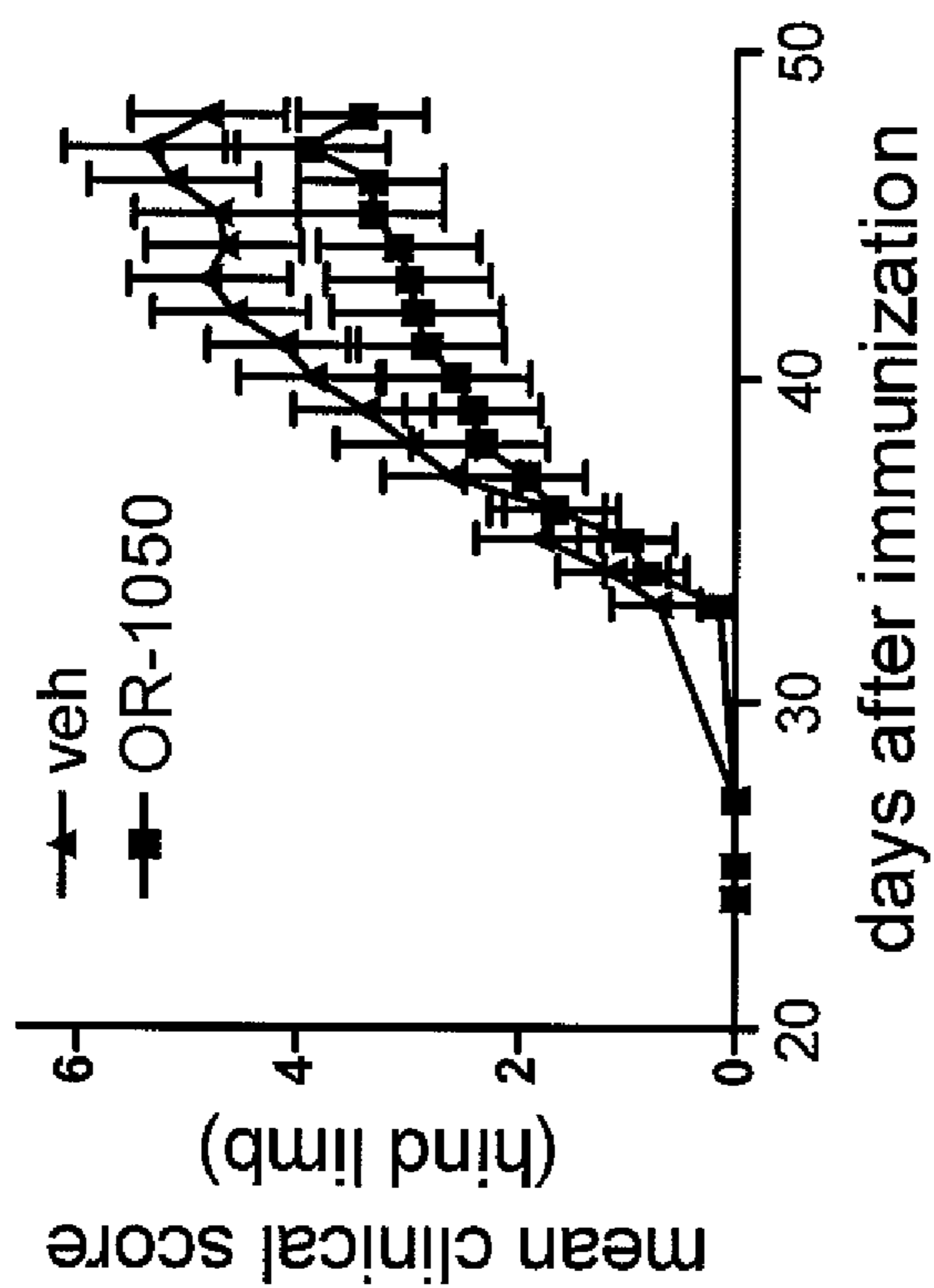


FIG. 17A

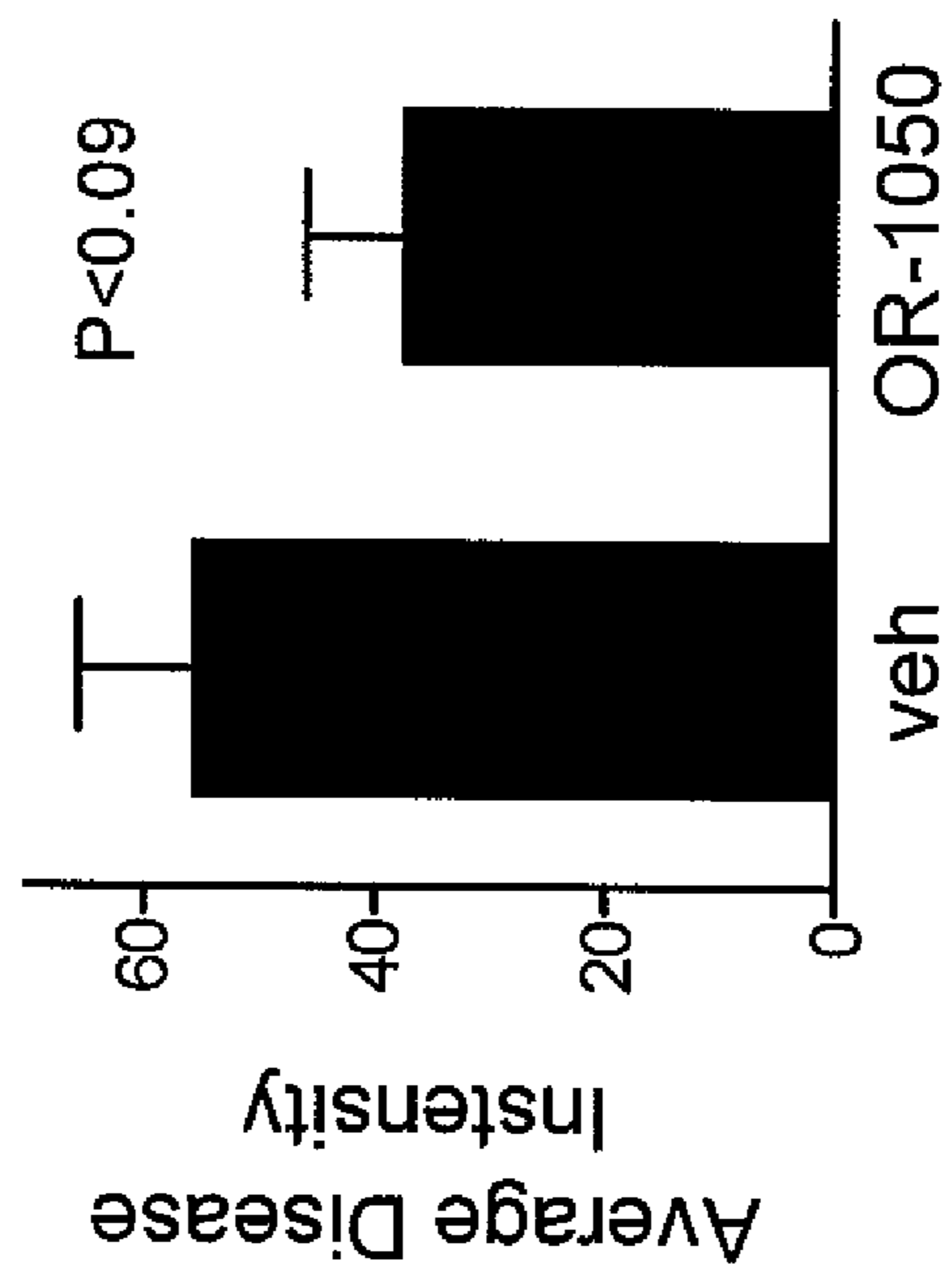


FIG. 17B

## MODULATORS OF RETINOID-RELATED ORPHAN RECEPTOR GAMMA

### RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 13/114,616, filed May 24, 2011, which is a continuation of U.S. application Ser. No. 11/867,637, filed Oct. 4, 2007, which claims priority under 35 U.S.C. §119(e) to U.S. Provisional Application No. 60/849,903, filed Oct. 5, 2006. The contents of these applications are incorporated herein by reference in their entireties.

### STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant Numbers 1 R43AI060447-01, 1 R03NS 050879-01, 1 R43DK071461-01, 1 R43 MH075461-01, 1 R43 NS 059219-01, 1 R43CA099875-01A1, and 1 R43AR055427-01 from the National Institutes of Health. The government has certain rights in the invention.

### REFERENCE TO SEQUENCE LISTING

The present application is being filed along with a sequence listing in electronic format. The sequence listing is provided as a file entitled ORPH.001C2.txt, created Feb. 13, 2013 which is 12 KB in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

### FIELD OF THE INVENTION

The present invention relates to methods for modulating (inhibiting or stimulating) retinoid-related orphan receptor  $\gamma$  (ROR $\gamma$ ) activity. This modulation has numerous effects, including inhibition of T<sub>H</sub>-17 cell function and/or T<sub>H</sub>-17 cell activity, and inhibition of re-stimulation of T<sub>H</sub>-17 cells, which are beneficial to treatment of inflammation and autoimmune disorders. In contrast, stimulation of ROR $\gamma$  results in stimulation of T<sub>H</sub>-17 cell function and/or activity which is beneficial for immune-enhancing compositions (e.g., vaccines). More specifically, the present invention relates to methods for inhibiting differentiation of T cells into T<sub>H</sub>-17 cells, or inhibiting the activity of T<sub>H</sub>-17 cells and other T cells that express IL-17, by contacting a population of T cells that may include T<sub>H</sub>-17 cells, with an antagonist of ROR $\gamma$ .

### BACKGROUND OF THE INVENTION

Many forms of serious human disease result from an autoimmune attack on the body. Many forms of autoimmune disease, such as rheumatoid arthritis, multiple sclerosis, psoriasis, and Crohn's disease, are particularly difficult to treat. An activated CD4<sup>+</sup> T cell that releases interleukin-17 (IL-17), a powerful pro-inflammatory cytokine (Aggarwal, Ghilardi et al. 2003), and is referred to as a T<sub>H</sub>-17 cell (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006), may be a significant pathogenic factor in autoimmune disease, based on observations that patients have high levels of IL-17 expression in target tissues. For example, the level of IL-17 mRNA was found to be elevated in brain autopsy tissue from patients with MS compared with controls, and the number of IL-17 positive mononuclear cells was also increased in the cerebrospinal fluid of affected patients (Matusevicius, Kivisakk

et al. 1999; Lock, Hermans et al. 2002; Vaknin-Dembinsky, Balashov et al. 2006). Rheumatoid arthritis (RA) also appears to involve a T cell-mediated autoimmune reaction. The level of IL-17 mRNA in synovial fluid of rheumatoid arthritis patients is predictive of disease progression (Kirkham, Lassere et al. 2006). Furthermore, T<sub>H</sub>-17 cells have been isolated from intestinal biopsies of patients with Crohn's Disease (Annunziato, Cosmi et al. 2007), and their frequencies are elevated in comparison to T cells isolated from normal intestine or peripheral blood. In disease tissue of psoriasis patients, levels of IL-22, another proinflammatory cytokine produced by T<sub>H</sub>-17 cells, are elevated (Zheng, Danilenko et al. 2007).

T<sub>H</sub>-17 cells, also referred to as IL-17<sup>+</sup>CD4<sup>+</sup> T or T<sub>IL-17</sub> cells, have only been recently characterized and are distinct from the T<sub>H</sub>1 and T<sub>H</sub>2 lineages of CD4<sup>+</sup> effector T cells. The relationship of T<sub>H</sub>-17 cells to the T<sub>H</sub>1 and T<sub>H</sub>2 lineages is diagrammed in FIGS. 1. T<sub>H</sub>1 and T<sub>H</sub>2-derived cytokines (such as IFN- $\gamma$  and IL-4) block T<sub>H</sub>-17 formation (FIG. 1) and targeted deletion of some transcription factors considered to be central for maintenance of the T<sub>H</sub>1 and T<sub>H</sub>2 phenotype have no major effect on T<sub>H</sub>-17 differentiation (Harrington, Hatton et al. 2005; Park, Li et al. 2005). IL-23 expression in mouse is required for induction of T cell-derived IL-17, and in culture IL-23 seems to act as a survival factor for pre-existing T<sub>H</sub>-17 cells but does not stimulate T<sub>H</sub>-17 formation from naïve CD4<sup>+</sup> T cells. Instead, the combination of TGF $\beta$  and IL-6 is required to stimulate the formation of T<sub>H</sub>-17 cells from naive mouse CD4<sup>+</sup> T cells, and there is genetic evidence in mouse for the involvement of IL-6 and TGF $\beta$  in T<sub>H</sub>-17 formation in vivo (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006). The cytokine requirements for T<sub>H</sub>-17 differentiation in human are somewhat different that in mouse but the T<sub>H</sub>-17 phenotype is similar, including expression of IL-17, IL-22, and ROR $\gamma$  (Annunziato, Cosmi et al. 2007; Kebir, Kreymborg et al. 2007).

The older view that autoreactive CD4<sup>+</sup> T<sub>H</sub>1 cells are a prime cause for the development or progression of MS and other forms of autoimmune disease has been challenged (Steinman 2007) by recent results from rodent models showing that mice with a targeted deletion in the p35 subunit of IL-12, which is required for the formation of T<sub>H</sub>1 cells, are still highly susceptible to the induction of experimental autoimmune encephalomyelitis (EAE) after immunization with myelin antigens (Becher, Durell et al. 2002; Gran, Zhang et al. 2002). In contrast, the knockout of either p19, the unique subunit of the cytokine IL-23, or p40, a common subunit of the two cytokines IL-12 and IL-23, create mice that are resistant to the induction of EAE (Cua, Sherlock et al. 2003; Langrish, Chen et al. 2005).

In addition to EAE, development of disease in a mouse model of rheumatoid arthritis, collagen-induced arthritis (Courtenay, Dallman et al. 1980), is also dependent on IL-23 function and is correlated with an increased level of T<sub>H</sub>-17 cell activity, such as release of IL-17 in affected tissues (Murphy, Langrish et al. 2003; Sato, Suematsu et al. 2006). Inhibition of IL-17 may be a viable alternative to inhibition of TNF $\alpha$  in treatment of RA (Lubberts, Koenders et al. 2005). Further, IL-23 expression is required for induction of IBD in mouse, and T<sub>H</sub>-17 cells appear to be an important downstream mediator of IL-23 effect in this model (Yen, Cheung et al. 2006). Disease progression in EAE models is partially suppressed in IL-17<sup>-/-</sup> mice or after treatment with anti-IL-17 antibodies (Iwakura and Ishigame 2006). IL-17<sup>-/-</sup> mice also show reduced incidence and severity in a collagen-induced arthritis (CIA) model for rheumatoid



arthritis and IL-17 antibodies have been shown to attenuate development of intestinal inflammation in rodent models of IBD (Nakae, Nambu et al. 2003; Yen, Cheung et al. 2006). Further, purified autoreactive  $T_H$ -17 cells strongly induce encephalomyelitis when transferred to naive mice, leading to more dramatic disease manifestation compared to disease induction by  $T_H$ 1CD4<sup>+</sup> T cells (Langrish, Chen et al. 2005; Komiyama, Nakae et al. 2006). The IL-27 receptor is prominently expressed in  $T_H$ -17 cells and the action of IL-27 as an inhibitor of murine EAE is correlated to reduced  $T_H$ -17 cell number in the animal (Batten, Li et al. 2006; Stumhofer, Laurence et al. 2006). Therefore, the activity of murine  $T_H$ -17 cells is well correlated to disease.

Inhibition of the human equivalent of the mouse  $T_H$ -17 cell is a highly desirable target for new therapeutic agents to treat autoimmune disease. Recent findings implicate  $T_H$ -17 cells in the pathogenesis of Crohn's disease and more generally inflammatory bowel disease (IBD). Antibody to the common subunit of IL-23 and IL-12, p40, is safe and may be clinically effective in treatment of Crohn's disease (Mannon, Fuss et al. 2004). A recent Phase 2 clinical trial also shows that anti-p40 is highly effective in the treatment of psoriasis (Krueger, Langley et al. 2007). The identification of  $T_H$ -17 cells has not only provided insight into autoimmune pathogenesis, it has also revealed a major pathway of adaptive immunity for extracellular microbes (Mangan, Harrington et al. 2006; Annunziato, Cosmi et al. 2007). Stimulation of  $T_H$ -17 differentiation, function and cytokine release therefore has the potential to enhance protective immunity by increasing T cell reactivity to pathogenic organisms and other targets, such as cancer-associated antigens.

#### Structure and Function of ROR $\gamma$

ROR $\gamma$  (NR1F3), a ligand-regulated nuclear transcription factor from the steroid/retinoid/thyroid family (Jetten, Kurebayashi et al. 2001), has been shown to be essential for CD4<sup>+</sup>  $T_H$ -17 development and/or function. ROR $\gamma$  participates in and is required for the development of  $T_H$ -17 cells.  $T_H$ -17 cells are absent from genetically-engineered mice that fail to express a specific splicing isoform of ROR $\gamma$ , ROR $\gamma$ t (Ivanov, McKenzie et al. 2006; Littman and Eberl 2006). Furthermore, an ROR $\gamma$ t-GFP transgene that expresses GFP from the ROR $\gamma$ t promoter is expressed in CD4<sup>+</sup>IL-17<sup>+</sup> T cells from the lamina propria of the gut and other tissues. In cell culture, transfection of ROR $\gamma$ t into naïve murine CD4<sup>+</sup> T cells induces differentiation of these cells into IL-17 expressing T cells even in the absence of the inducing cytokines IL-6 and TGF $\beta$ . The data suggest that the transcriptional activity of ROR $\gamma$ t is of major importance to  $T_H$ -17 cell differentiation and function (Ivanov, McKenzie et al. 2006). ROR $\gamma$ t expression is induced in the presence of TGF $\beta$  and IL-6 or by TGF $\beta$  and IL-21, an autocrine cytokine released from developing  $T_H$ -17 cells in response to IL-6 (Ivanov, McKenzie et al. 2006; Korn, Bettelli et al. 2007; Nurieva, Yang et al. 2007; Zhou, Ivanov et al. 2007).

The expression of ROR $\gamma$  follows a similar pattern in human as in mouse T cells: ROR $\gamma$  is more highly expressed in IL-17 or IL-17/IFN $\gamma$  expressing CD4<sup>+</sup> T cells (Th-17) than in CD4<sup>+</sup> T cells that express IFN $\gamma$  alone (Th-1) (Acosta-Rodriguez, Napolitani et al. 2007; Acosta-Rodriguez, Rivino et al. 2007; Annunziato, Cosmi et al. 2007; Wilson, Boniface et al. 2007). Finally, it has been reported that other murine T cell types express ROR $\gamma$ t, including  $\gamma\delta$ TCR<sup>+</sup> cells, CD8<sup>+</sup> T cells, and iNKT cells (Ivanov, McKenzie et al. 2006; Ivanov and Littman 2007). Since these cells are also express

IL-17, it is possible that ROR $\gamma$ t is required for the differentiation of the IL-17<sup>+</sup> subpopulations of several other types of T cells.

Finally, in the absence of ROR $\gamma$ t, mice are much less susceptible to the induction of EAE (Ivanov, McKenzie et al. 2006). These studies were carried out in immunodeficient mice. ROR $\gamma$ t<sup>-/-</sup> mice lack lymph nodes, and although they are resistant to the development of EAE, a further study was carried out by adoptive transfer of ROR $\gamma$ t wild type or ROR $\gamma$ t<sup>-/-</sup> bone marrow to immunodeficient mice. While transfer of normal bone marrow rendered the mice sensitive to the induction of EAE by peptide immunization, the ROR $\gamma$ t<sup>-/-</sup> transfectants were substantially resistant (Ivanov, McKenzie et al. 2006). These data suggest that ROR $\gamma$ t mediated regulation of T cell differentiation is involved in the development of autoimmune disease.

**ROR $\gamma$  Structure.** ROR $\gamma$ , like other members of the nuclear receptor family, has a bipartite structure with two major functional domains, a DNA binding domain (DBD) and a ligand binding domain (LBD, see FIG. 2) (Medvedev, Yan et al. 1996). Ligand-regulated transcription of the nuclear receptors is mediated through the LBD, which has been crystallized for many receptors (Li, Lambert et al. 2003), including ROR $\alpha$  and ROR $\beta$ , the receptors most closely related to ROR $\gamma$ . The ROR $\gamma$  LBD is predicted to have a binding pocket similar to ROR $\alpha$  and ROR $\beta$  (Stehlin, Wurtz et al. 2001; Kallen, Schlaeppli et al. 2004). The retinoic acid receptors (RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$ ) are more distantly related and appear not to have a functional overlap with the RORs (Jetten, Kurebayashi et al. 2001).

The nuclear receptor LBD recruits transcriptional coregulators (FIG. 2) in response to small molecule compounds (Li, Lambert et al. 2003; Savkur and Burris 2004). These coregulatory proteins act as sensors for the conformational state of the ligand-bound complex and in turn regulate the recruitment of transcriptional factors to chromatin adjacent to the receptor. Short peptide domains of the coregulatory proteins required for interaction with the nuclear receptor contain the conserved sequence LXXLL (SEQ ID NO: 1), and synthetic peptide recruitment assays based on this motif are widely used to monitor the binding of agonists and antagonists to the nuclear receptor LBD (Lee, Elwood et al. 2002; Savkur and Burris 2004), including for an assay described herein for ROR $\gamma$ .

**Functional Studies of ROR $\gamma$ .** Recognition of specific DNA motifs by the nuclear receptor DBD determines specificity for gene transcription; however, little is known about specific gene targets for ROR $\gamma$ , and the major findings on ROR $\gamma$  function have been derived from studies of gene-targeted mice (Kurebayashi, Ueda et al. 2000; Sun, Unutmaz et al. 2000; Eberl and Littman 2004; Eberl, Marmon et al. 2004). ROR $\gamma$  has two splicing isoforms, ROR $\gamma$  and ROR $\gamma$ t (He, Deftos et al. 1998). ROR $\gamma$ t differs from ROR $\gamma$  by a truncation of 21 amino acids at the N-terminal and is the isoform specifically expressed in thymus, lymph node precursors, and  $T_H$ -17 cells (Eberl and Littman 2004; Eberl, Marmon et al. 2004; Ivanov, McKenzie et al. 2006; Littman and Eberl 2006), the major tissues affected in knockout studies of ROR $\gamma$ . The significance of the N-terminal deletion of ROR $\gamma$  is not known, but it is unlikely to affect ligand specificity or LBD function, which is encoded at the receptor's C-terminal and is identical in the two splicing isoforms.

In addition to its requirement for  $T_H$ -17 differentiation, ROR $\gamma$  has other discrete functions in the immune system. In its absence, the survival of the major subtype of developing T lymphocytes, the CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) thymocytes, is reduced (Kurebayashi, Ueda et al. 2000; Sun,

Unutmaz et al. 2000), and the embryonic formation of lymph nodes and Peyer's patches is blocked (Eberl, Marmon et al. 2004). ROR $\gamma$ t is an early marker for the embryonic formation of lymphoid tissue inducer or LTi cells. LTi cells are involved in lymph node and Peyer's patch formation during embryogenesis (Eberl, Marmon et al. 2004). After birth, an LTi-like cell participates in formation of intermediate lymphoid follicles (ILFs) of the gut. These lymphoid structures appear to participate in the gut immune response (Eberl and Littman 2004). ROR $\gamma$  is also expressed in liver, muscle, and fat (Jetten, Kurebayashi et al. 2001; Fu, Sun et al. 2005). A recent study of ROR $\gamma^{-/-}$  mice also suggests that the receptor has some regulatory effects on Phase 1 and Phase 2 detoxification enzymes (Kang, Angers et al. 2007).

**Clinical Significance.** Three major points of action of ROR $\gamma$  in the immune system have been identified in gene knockout studies; T cells, including the T<sub>H</sub>-17 cell, lymph node formation, and survival of DP thymocytes. Of these, regulation of IL-17<sup>+</sup> T cell function, including T<sub>H</sub>-17 differentiation, appears to be most relevant to human therapeutics. Not only is the receptor absolutely required for T<sub>H</sub>-17 differentiation, but the supply of pathogenic T<sub>H</sub>-17 cells must be constantly replenished since they are destroyed in target tissue (Gold, Lington et al. 2006). Inhibition of new T<sub>H</sub>-17 formation will therefore have important benefits. ROR $\gamma$  is expressed in human memory T<sub>H</sub>-17 cells (Acosta-Rodriguez, Rivino et al. 2007; Annunziato, Cosmi et al. 2007), and data presented herein shows that ROR $\gamma$  antagonists block IL-17 expression in human peripheral blood mononuclear cells (PBMCs). The primary source of IL-17 in PBMCs has been reported to be memory T cells (Shin, Benbernou et al. 1999). Finally, ROR $\gamma$  enhances, but is not absolutely required for, the survival of the major developing T cell type of the thymus, the DP thymocyte. In the knockout animal, there is no evidence of immunodeficiency although splenic and peripheral frequencies of some immune cell types are changed. However, there is a much higher rate of apoptosis among thymocytes and thymocyte number is reduced (Kurebayashi, Ueda et al. 2000; Zhang, Guo et al. 2003). The thymus of an adult has already atrophied to a considerable degree and can be reversibly suppressed by many forms of pharmacological treatment, including exposure to steroids (Haynes, Markert et al. 2000). These data suggest that secondary effects of an ROR $\gamma$  antagonist on thymic function in human may not be clinically significant.

Prior to 1990, no drugs to treat MS were available. In the last 15 years, several treatment options have emerged, primarily various forms of INF $\beta$  (Avonex, Rebif, Betaseron), Glatiramer acetate (Copaxone) and the chemotherapeutic drug mitoxantrone (Novantrone) (Rolak 2003). INF $\beta$  and Glatiramer acetate both appear to inhibit T cell activation and both drugs reduce the number of attacks in relapsing-remitting MS but have little effect in the progressive phase of the disease (Dhib-Jalbut 2002; Rolak 2003). The long-term benefits of these drugs are unclear. Mitoxantrone appears to retard progression and delay disability in secondary progressive MS. However, toxicity of this drug is very limiting and Mitoxantrone is considered a short-term treatment option. More recently, a humanized antibody to  $\alpha$ 4 $\beta$ 1 integrin (NATALIZUMAB, Tysabri) has been approved for the treatment of MS and has been shown to slow disease progression and reduce relapse rate in several clinical trials using different outcome measures (Steinman 2005). Compared to existing treatments, the efficacy of Tysabri is quite dramatic. However, several cases of progressive multifocal leukoencephalopathy (PML), a lethal resurgence of a latent viral infection linked to the immunosuppressive action of the

drug, led to withdrawal (Rudick, Stuart et al.). Tysabri has now been reintroduced into the market with much stricter patent monitoring. Tysabri likely blocks both T<sub>H</sub>-17 and T<sub>H</sub>-1 cells. A novel small molecule drug that is specific for T<sub>H</sub>-17 cells and does not compromise the antiviral activity of T<sub>H</sub>-1 cells could be safer and more effective for several reasons: (1) a small molecule drug, administered daily, can be rapidly withdrawn if significant side effects occur; (2) a small molecule drug is more readily manufactured and more easily administered than a biologic, such as Tysabri; and (3) T<sub>H</sub>-1 cells suppress T<sub>H</sub>-17 differentiation and thus specific inhibition of T<sub>H</sub>-17 cells may be more effective.

A number of other small molecule drugs are marketed for autoimmune diseases such as rheumatoid arthritis, IBD, and psoriasis. Many of these, such as methotrexate or azathioprine, carry significant toxicity because of anti-metabolite or anti-mitotic effects. Dosing is usually limited. A small molecule drug that has a more specific mechanism of action, such as inhibition of T<sub>H</sub>-17 cells or other IL-17 expressing T cells, are likely to be safer and, hence, more efficacious as dosages may be elevated to have a substantial inhibitory effect on target cells.

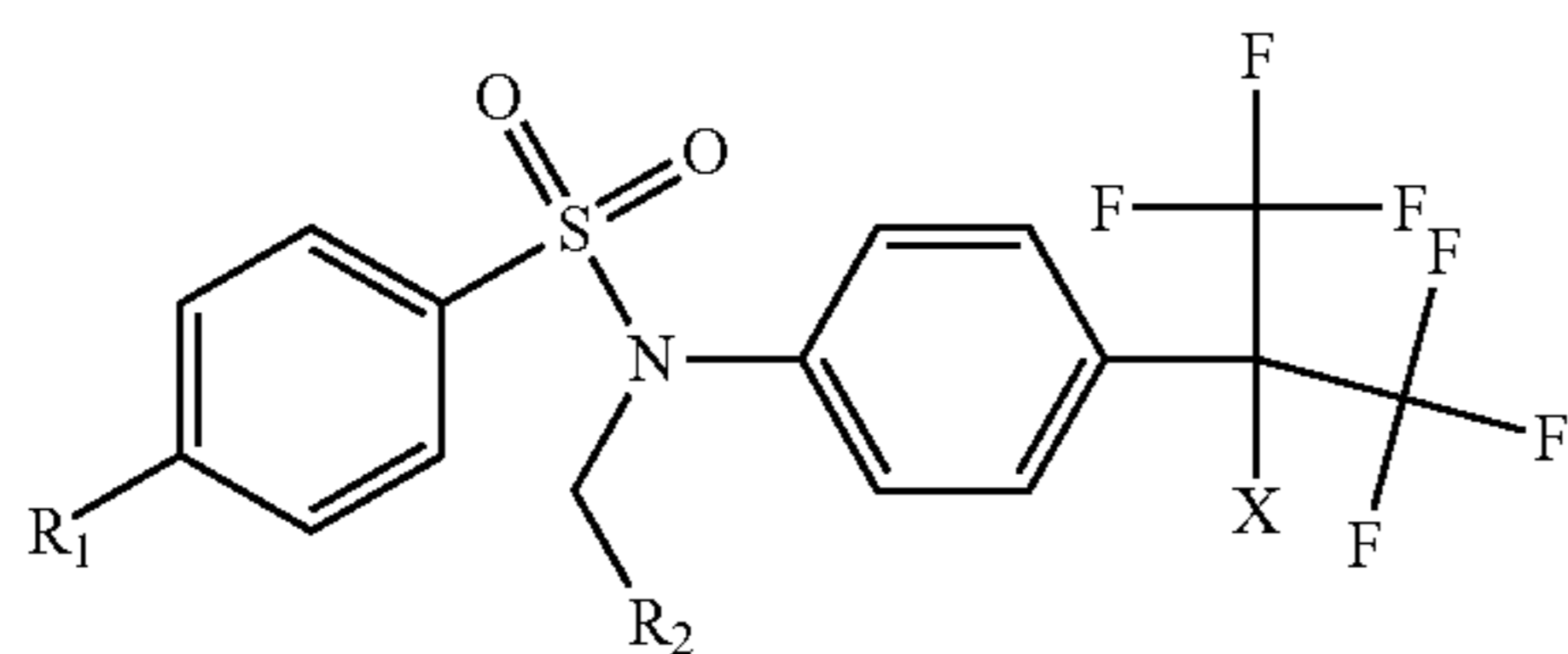
Pharmacologically useful ligands to members of the ROR family of orphan nuclear receptors have not been identified in the published literature. Cholesterol and cholesterol sulfate occupy the ligand binding pocket within the receptor LBD of ROR $\alpha$  as determined by x-ray crystallography (Kallen, Schlaeppli et al. 2002; Kallen, Schlaeppli et al. 2004), but, due to the fact that these molecules are plentiful in normal cells, it has not been possible to use these molecules in order to characterize ROR $\alpha$  as a pharmacological target (Moraitis and Giguere 2003). A series of ROR $\alpha$  ligands was published in 1996 (Missbach, Jagher et al. 1996; Wiesenberg, Chiesi et al. 1998), but these findings have not been independently replicated or evaluated by functional criteria described below. One of the proposed ligands for ROR $\beta$ , melatonin, has been challenged in the literature (Becker-Andre, Wiesenberg et al. 1994; Greiner, Kirfel et al. 1996; Becker-Andre, Wiesenberg et al. 1997). More recently, it was proposed that all-trans retinoic acid and the synthetic retinoid ALRT 1550 are functional ligands for ROR $\beta$  and that these two ligands also regulate ROR $\gamma$  in a similar manner, in both cases inhibiting the transcriptional activity of the receptors. All-trans retinoic acid and ALRT 1550 were referred to as "functional" ligands because they were presumed to both bind and regulate transcription through ROR $\beta$ . The ligands are unlikely to be useful pharmacologically because they are potent activators of the retinoid receptors, RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$  (Thacher, Vasudevan et al. 2000). Therefore all of these ligands fail the test of functional usefulness either on the criterion that their effects have not been reproducible, or because they are ubiquitous, or because they lack specificity. This application describes assay for ROR $\gamma$ , and ROR $\gamma$  ligands that have pharmacologically useful potency (in the range of 50 nM to 1  $\mu$ M), that have good selectivity, as demonstrated by assays for other members of the nuclear receptor family. These ligands, both agonists and antagonists, have drug-like properties as indicated by rational structure activity relationships among analogues as well as other drug-like properties such as bioavailability and activity in cellular and animal models. Specific ROR $\gamma$  antagonists are predicted to be highly useful in treatment of autoimmune disease by blocking T<sub>H</sub>-17 function. Agonists of ROR $\gamma$  are predicted to enhance immu-

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nity and to have application in stimulation of vaccination and in adjuvant cancer therapy.

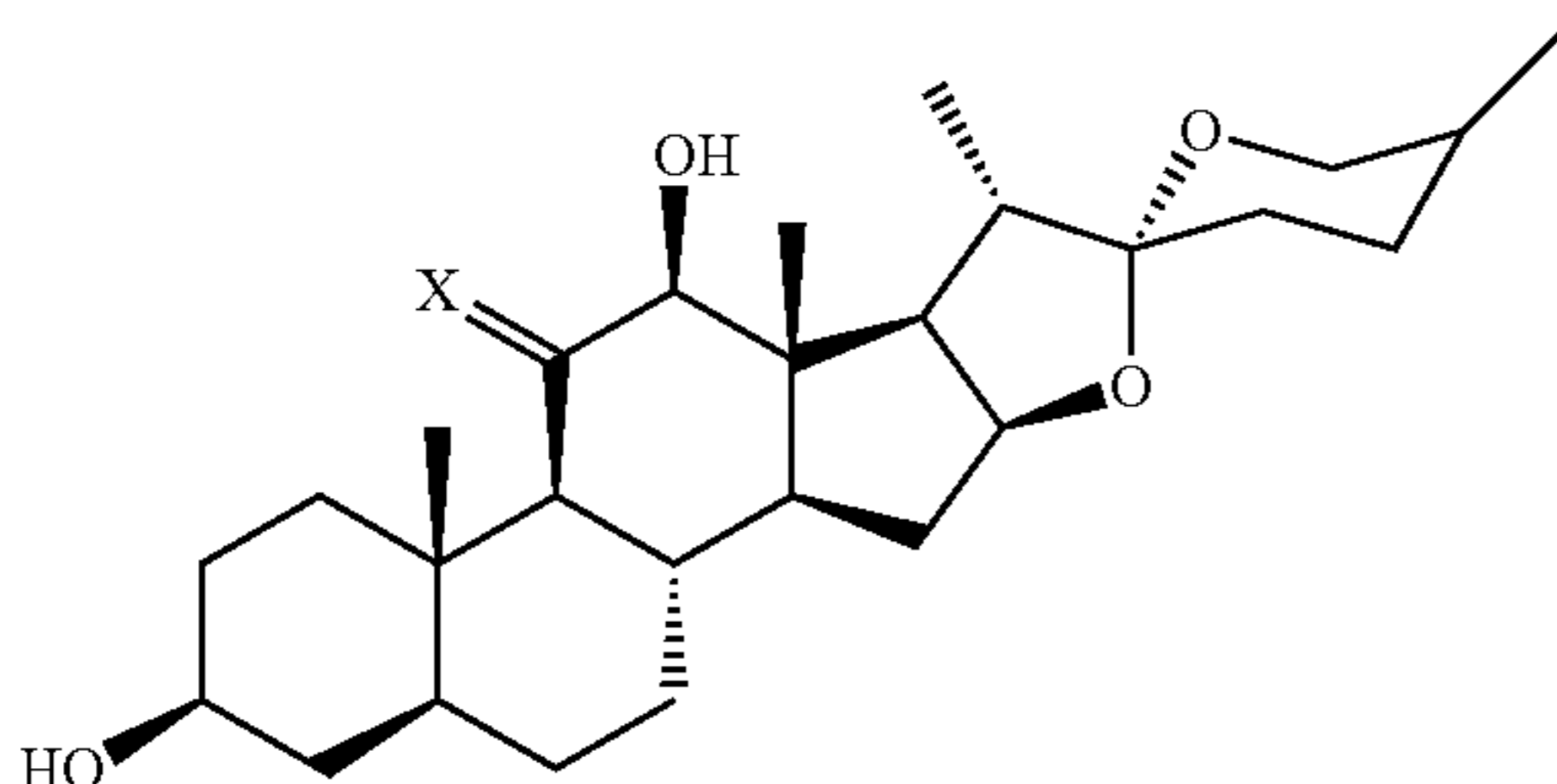
## SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting  $T_H$ -17 cell differentiation from naïve T cells, or  $T_H$ -17 cell function/activity, comprising contacting a population of T cells that may include  $T_H$ -17 cells with an effective amount of an antagonist of retinoic acid related orphan receptor  $\gamma$  (ROR $\gamma$ ). In one embodiment,  $T_H$ -17 cell function/activity is release of a cytokine. The cytokine may be interleukin-17 or interleukin-22. In one embodiment, the ROR $\gamma$  antagonist is at least 20-fold more potent as an ROR $\gamma$  antagonist than as an LXR agonist. In another embodiment, the  $T_H$ -17 cell differentiation or cytokine release is associated with an inflammatory or an autoimmune disorder. In another embodiment, the inflammatory or autoimmune disorder is arthritis, diabetes, multiple sclerosis, uveitis, rheumatoid arthritis, reactive arthritis, sarcoidosis, psoriasis, psoriatic arthritis, asthma, bronchitis, allergic rhinitis, chronic obstructive pulmonary disease, atherosclerosis, *H. pylori* infections, ulcers resulting from *H. pylori* infections, inflammatory bowel disease, Crohn's Disease ulcerative colitis or sprue. Preferably, the ROR $\gamma$  antagonist is a small molecule drug, and is preferably not a polynucleotide. In one embodiment, the antagonist has the structure:



wherein  $R_1$ =H,  $C_1$ - $C_6$  alkyl, F, Cl, Br, I,  $NO_2$ ;  $R_2$ = $C_1$ - $C_4$  alkyl; and  $X$ =OH, or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof.

In one embodiment, the antagonist is OR-1050. In another embodiment, the antagonist has the structure:



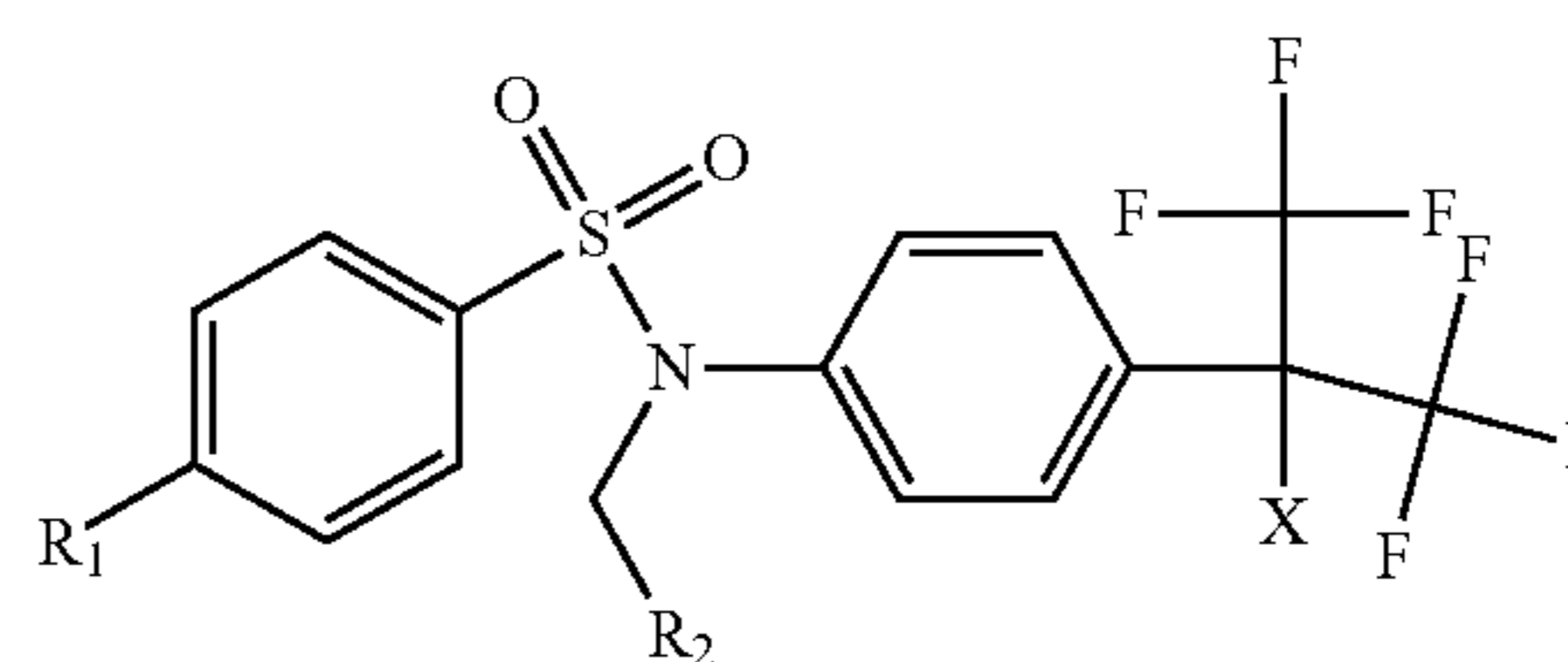
wherein  $X$ =O or is absent ( $X$ =H,H), or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof.

In other embodiments, the antagonist is OR-885, OR-345, OR-13571, OR-2161, OR-133171, or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof.

The present invention also provides a method of inhibiting  $T_H$ -17 cell differentiation from naïve T cells, or  $T_H$ -17 cell function/activity, in an individual in need thereof, comprising administering an effective amount of an ROR $\gamma$  antagonist to the individual. In one embodiment, the  $T_H$ -17 cell function/activity is release of a cytokine. In one embodi-

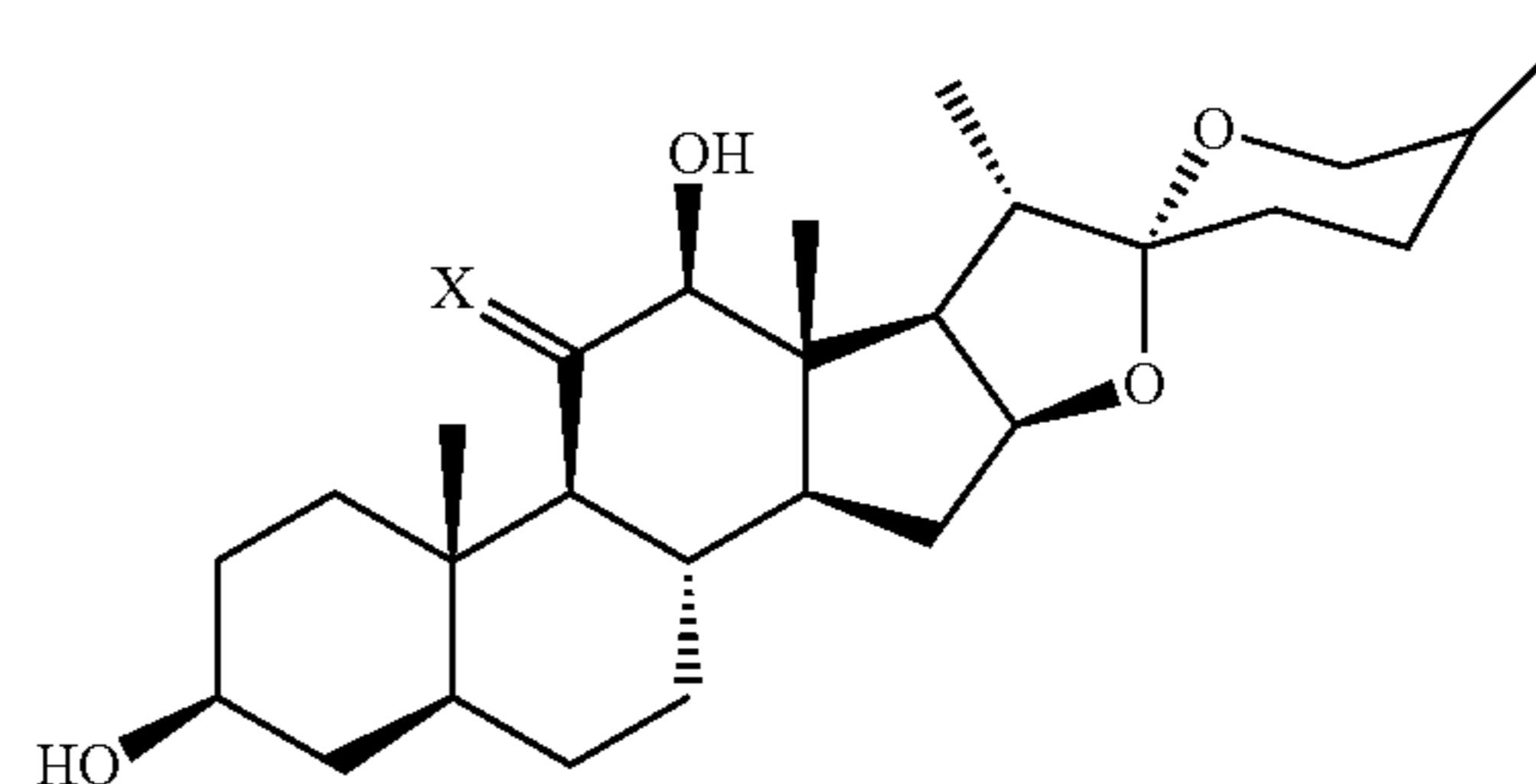
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ment, the cytokine is interleukin-17 or interleukin-22. In another embodiment, the ROR $\gamma$  antagonist is at least 20-fold more potent as ROR $\gamma$  antagonist than as LXR agonist. In one embodiment, the ROR $\gamma$  antagonist is orally administered. In another embodiment, the ROR $\gamma$  antagonist is administered on a daily basis without resulting in weight loss or hypertriglyceridemia. In another embodiment, the  $T_H$ -17 cell differentiation or cytokine release is associated with an inflammatory or autoimmune disorder. In another embodiment, the inflammatory or autoimmune disorder is arthritis, diabetes, multiple sclerosis, uveitis, rheumatoid arthritis, reactive arthritis, sarcoidosis, psoriasis, psoriatic arthritis, asthma, bronchitis, allergic rhinitis, chronic obstructive pulmonary disease, atherosclerosis, *H. pylori* infections, ulcers resulting from *H. pylori* infections or inflammatory bowel disease, Crohn's Disease, ulcerative colitis, or sprue. In one embodiment, the antagonist has the structure:



wherein  $R_1$ =H,  $C_1$ - $C_6$  alkyl, F, Cl, Br, I,  $NO_2$ ;  $R_2$ = $C_1$ - $C_4$  alkyl; and  $X$ =OH, or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof.

In one embodiment, the antagonist is OR-1050. In another embodiment, the antagonist has the structure:



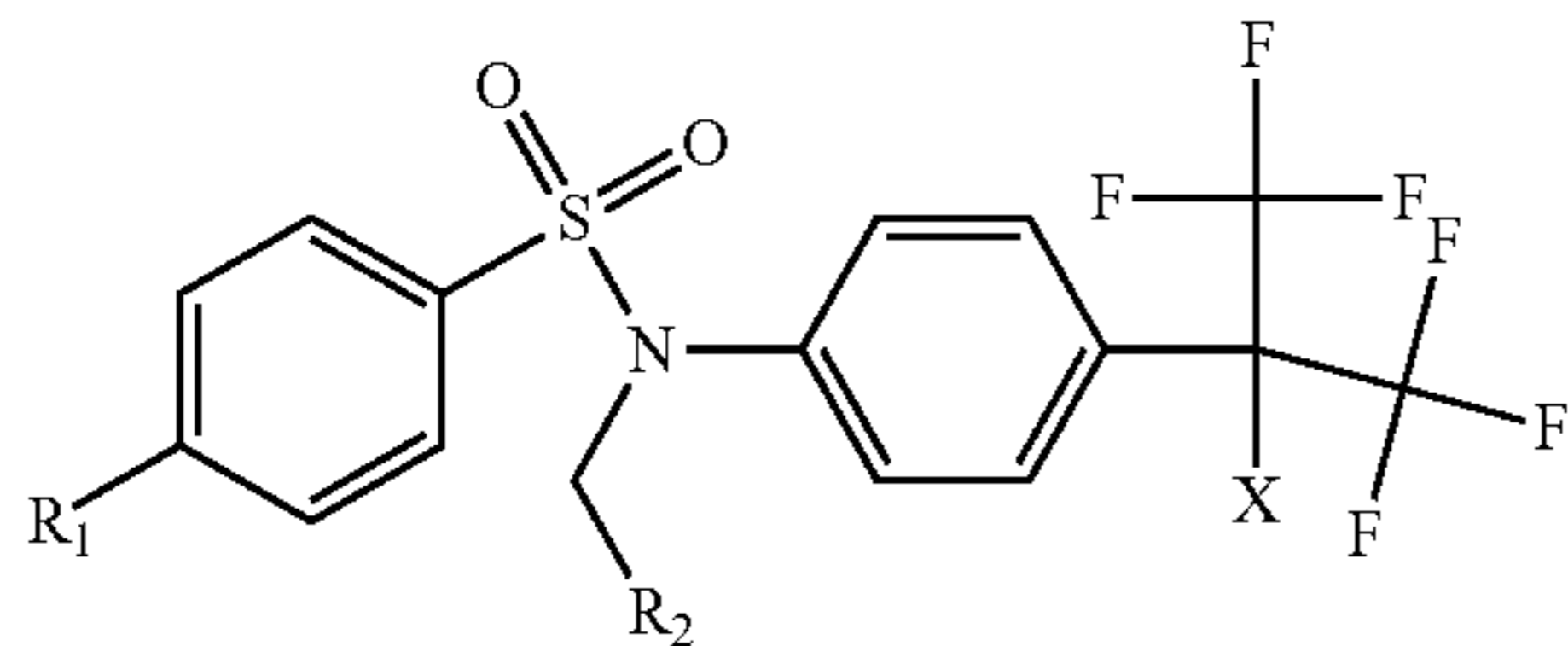
wherein  $X$ =O or is absent ( $X$ =H,H), or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof.

In another embodiment, the antagonist is OR-885, OR-345, OR-13571, OR-2161, OR-133171 or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof.

The present invention also provides a method of treating an inflammatory or autoimmune disease in an individual, comprising identifying an individual in need of such treatment, and administering an effective amount of an ROR $\gamma$  antagonist to the individual. In another embodiment, the ROR $\gamma$  antagonist is at least 20-fold more potent as ROR $\gamma$  antagonist than as LXR agonist. In one embodiment, the ROR $\gamma$  antagonist is orally administered. In another embodiment, the ROR $\gamma$  antagonist is administered on a daily basis without resulting in weight loss or hypertriglyceridemia. In one embodiment, the inflammatory or autoimmune disorder is arthritis, diabetes, multiple sclerosis, uveitis, rheumatoid arthritis, reactive arthritis, sarcoidosis, psoriasis, psoriatic arthritis, asthma, bronchitis, allergic rhinitis, chronic obstructive pulmonary disease, atherosclerosis, *H. pylori*

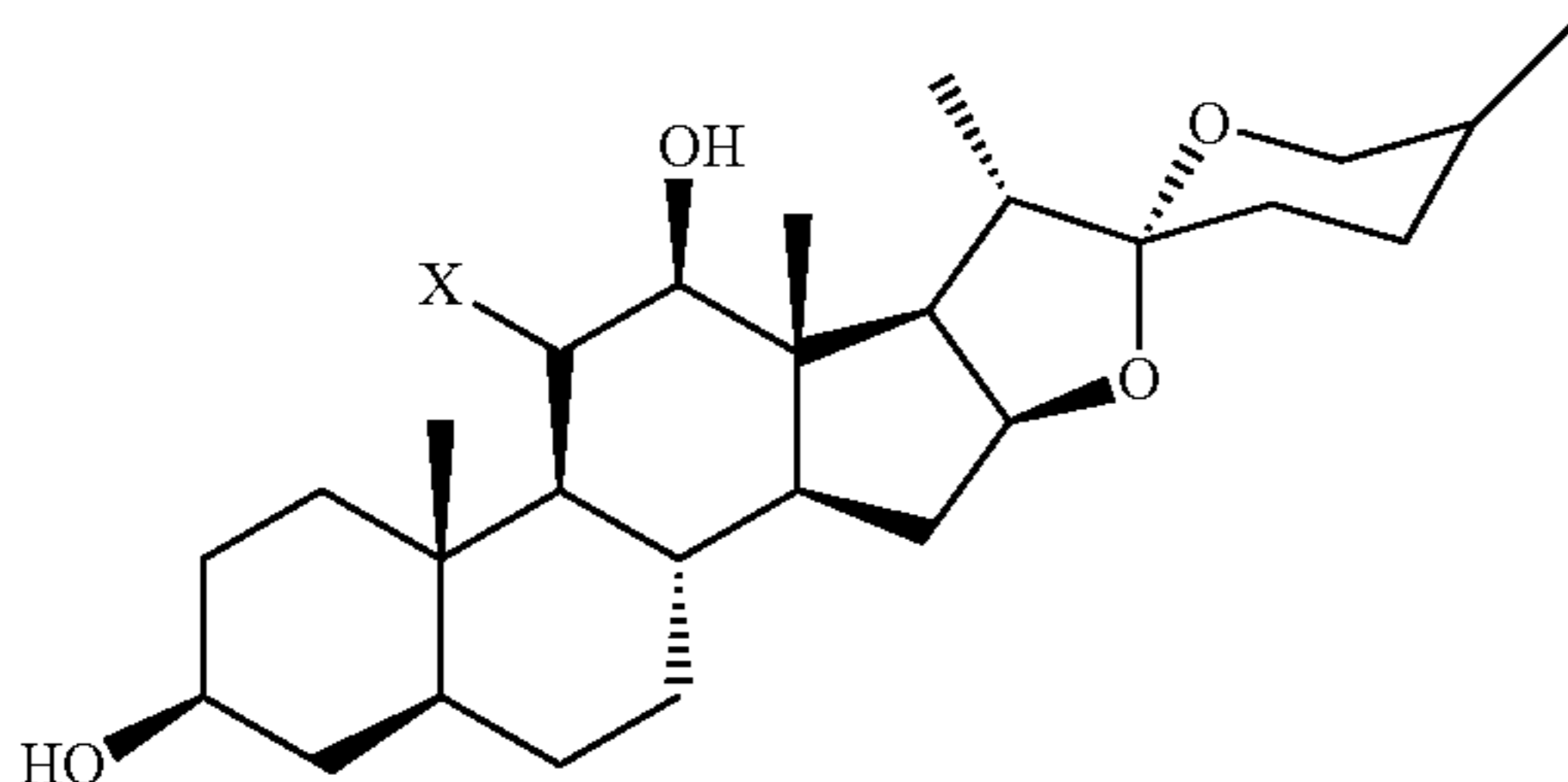
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infections, ulcers resulting from *H. pylori* infections or inflammatory bowel disease. In one embodiment, the inflammatory bowel disease is Crohn's disease, ulcerative colitis or sprue. Preferably, the ROR $\gamma$  antagonist is a small molecule drug. It is preferably not a polynucleotide, including DNA, antisense, siRNA, and the like. In one embodiment, the antagonist has the structure:



wherein R1=H, C1-C6 Alkyl, F, Cl, Br, I, NO<sub>2</sub>; R2=C1-C4 Alkyl; and X=OH, or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof.

In one embodiment, the antagonist is OR-1050. In another embodiment, the antagonist has the structure



wherein X=O or is absent (X=H,H), or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof. In other embodiments, the antagonist is OR-885, OR-345, OR-13571, OR-2161, OR-133171 or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof.

The present invention also provides a method of screening for agonists or antagonists to ROR $\alpha$ , ROR $\gamma$  or ROR $\beta$ , comprising contacting a compound with a labeled, expressed ROR LBD and a labeled peptide that includes residues 710-720 (RTVLQLLLGNP; SEQ ID NO: 2) of human RIP140; and measuring the proximity of the two labels, wherein binding of labeled peptide identifies the compound as an agonist and displacement of labeled peptide identifies the compound as an antagonist. In one embodiment, the proximity of the two labels is measured using radioactive or fluorescent probes. In another embodiment, the ROR LBD is labeled with glutathione-S-transferase (GST), and the peptide is labeled with biotin. In another embodiment, wherein the peptide has the sequence ERRT-VLQLLLGNSNK (SEQ ID NO: 3), wherein biotin is linked to the N-terminus of the peptide by an aminohexanoic acid linker.

The present invention also provides a method of increasing the number of T cells reactive to a specific antigen, comprising administering an ROR $\gamma$  agonist in conjunction with, or subsequent to, administration of the antigen.

The present invention also provides a method of increasing the immunogenicity of an immunogenic composition in an individual in need thereof, comprising administering an immunogenicity-increasing amount of an ROR $\gamma$  agonist in conjunction with, or subsequent to, the immunogenic com-

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position. In one embodiment, the immunogenic composition is a vaccine composition. In another embodiment, the vaccine composition is an attenuated live vaccine or a non-replicating and/or subunit vaccine, wherein the vaccine induces memory T<sub>H</sub>-17 cells specific for said vaccine. In one embodiment, the vaccine is a tumor vaccine, viral vaccine, bacterial vaccine or parasitic vaccine. In one embodiment, the viral vaccine is a DNA viral vaccine, an RNA viral vaccine or a retroviral viral vaccine.

The present invention also provides a method of increasing mucosal immunity to a preselected antigen, comprising administering to a subject a mucosal immunity-increasing amount of an ROR $\gamma$  agonist in conjunction with, or subsequent, to the antigen. In one embodiment, the antigen is a bacterial antigen, viral antigen or tumor antigen.

The present invention also provides a method of enhancing induction, expression and/or release of a pro-inflammatory cytokine, a pro-inflammatory cytokine receptor, a pro-inflammatory chemokine or a pro-inflammatory chemokine receptor in cells capable of expressing said cytokine, cytokine receptor, chemokine or chemokine receptor, comprising administering an ROR $\gamma$  agonist to the cells. In one embodiment, the ROR $\gamma$  agonist is a small organic molecule, protein, peptide, nucleic acid, carbohydrate or antibody. In another embodiment, the pro-inflammatory cytokine is IL-17 or IL-22. In one embodiment, the cells are contacted in vitro, ex vivo or in vivo.

The present invention also provides a method of inducing T<sub>H</sub>-17 cell differentiation and/or transcription of IL-17 and/or IL-22 in a population of T cells that may include T<sub>H</sub>-17 cells, comprising contacting the cells with an effective amount of an ROR $\gamma$  agonist. In one embodiment, the cells are contacted in vitro, ex vivo or in vivo.

The present invention also provides a method of inducing T<sub>H</sub>-17 cell differentiation in an individual in need thereof, comprising administering an effective T<sub>H</sub>-17 cell differentiation-inducing amount of an ROR $\gamma$  agonist to the individual.

The present invention also provides a method of inhibiting T<sub>H</sub>-17 cell differentiation and/or release of IL-17 and/or IL-22, comprising administering an effective amount of an ROR $\gamma$  antagonist to a population of T cells that may include T<sub>H</sub>-17 cells. In one embodiment, the antagonist is a small organic molecule, protein, peptide, nucleic acid, carbohydrate or antibody. In another embodiment, the T cell is a CD4<sup>+</sup> T cell, a CD8<sup>+</sup> T-cell or a TCR $\gamma$  $\delta$ <sup>+</sup> T cell. In one embodiment, the antagonist is administered in vitro, ex vivo or in vivo.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Naive T cell differentiation in mouse. T<sub>H</sub>-17 cell differentiation is independent of T<sub>H</sub>1 and T<sub>H</sub>2. IFN $\gamma$  and IL-4, from T<sub>H</sub>1 and T<sub>H</sub>2, respectively, antagonize T<sub>H</sub>-17 differentiation, while TGF $\beta$  and IL-6 stimulate T<sub>H</sub>-17 differentiation. ROR $\gamma$ t is required for T<sub>H</sub>-17 cell formation, and its expression appears to take place early in differentiation (Ivanov, McKenzie et al. 2006). IL-23 appears to have a stimulatory effect on the differentiated T<sub>H</sub>-17 cell.

FIG. 2. Nuclear Receptor Structure. The activation function-1 (AF-1) domain induces gene transcription independently of ligand. The activation function-2 domain (AF-2) is a part of the LBD and is required for the ligand-dependent transcriptional effect.

FIG. 3. Dose response of ROR $\gamma$  antagonists in a Chinese Hamster Ovary (CHO) cell assay of transcription. CHO cells were transfected with an expression plasmid encoding a

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fusion of the DNA-binding domain (DBD) of the yeast transcriptional factor Gal4 with the ligand-binding domain of mouse ROR $\gamma$  (Gal4-mROR $\gamma$ ). A luciferase reporter containing a 5 $\times$ Gal4 response element at its promoter region was cotransfected with the receptor chimera. T0901317 (Schultz, Tu et al. 2000) and OR-1050 are added in DMSO and each point is the median of triplicate values, normalized to a DMSO-only control.

FIGS. 4A-B. Identification and characterization of ROR $\gamma$  agonists. To lower the transcriptional basal activity of ROR $\gamma$  in CHO cells and increase the dynamic range for ROR $\gamma$  activation by agonists, one micromolar of the ROR $\gamma$  antagonist T0901317 (Cayman Chemical, Ann Arbor Mich.) was added to the cell-based assay described in FIG. 3. (FIG. 4A) OR-942 (hyodeoxycholic acid methyl ester) was identified as an agonist in the transcriptional assay. When OR-942 was tested alone, GAL4-ROR $\gamma$  transcriptional activity was only modestly elevated in CHO cells. In the presence of the antagonist T0901317, however, ROR $\gamma$  displays lowered basal activity and OR-942 activates ROR $\gamma$  transcriptional activity by approximately 8-fold.

(FIG. 4B) The agonist activity of OR-942 was confirmed in a biochemical assay based on coregulatory peptide recruitment. OR-942 induces an interaction between partially purified ROR $\gamma$  LBD, expressed as a fusion protein with glutathione-S-transferase (GST-ROR $\gamma$ ), and a 15-mer peptide (ERRTVLQLLLGNPTK; SEQ ID NO: 4), or peptide K, derived from the human coregulator protein RIP140 (Lee, Elwood et al. 2002). The peptide is biotinylated at the amino-terminal. The interaction of peptide K and GST-ROR $\gamma$  was followed by fluorescence resonance energy transfer (FRET). The two components of the assay were labeled with an anti-GST antibody coupled to allophycocyanin (APC) and streptavidin-R-phycoerythrin (SA-RPE). FRET units were calculated as the fractional increase ( $\times 100$ ) of the APC/RPE fluorescence ratio over the value obtained in the presence of a mutated form of the K peptide (Kmut, ERRTVLQLLVGNPTK; SEQ ID NO: 5), also biotinylated at the N-terminal amino acid residue, that substitutes two of the leucine residues required for coactivator peptide binding (Darimont, Wagner et al. 1998; Li, Lambert et al. 2003) with valine.

FIG. 5. The ROR $\gamma$  antagonist T0901317 has the properties of a competitive antagonist to OR-942. A dose response of the ROR $\gamma$  agonist OR-942 was carried out in the biochemical assay with GST-ROR $\gamma$  and peptide K. The effect of the antagonist T0901317 is to shift the estimated EC<sub>50</sub> of OR-942 from 0.4  $\mu$ M to 4  $\mu$ M, consistent with competition for a single binding site on ROR $\gamma$ . Each point is the median of triplicate values.

FIG. 6. Simultaneous characterization of an ROR $\gamma$  agonist and antagonist in the biochemical assay for ROR $\gamma$ . The activities of OR-1050 and OR-12872 were compared in a coregulatory peptide recruitment assay that uses peptide biotinylated K1 (from RIP140 of rat) instead of peptide K (from RIP140 of human). The sequence of peptide K1 is ERRTVLQLLLGNSNK (SEQ ID NO: 3). The mutated form of the K1 peptide (K1mut, ERRTVLQLLVGNSNK; SEQ ID NO: 6) was used as a control. The biotin was separated from the K1 and K1mut N-terminus by an amino-hexanoic acid linker. The combination of GST-ROR $\gamma$  and peptide K1 has a significant FRET value in the absence of added ligand, and this enables characterization of both agonist and antagonist in the same assay format. Values are the average of duplicate measurements.

FIG. 7. Regulation of T<sub>H</sub>-17 cell differentiation by ROR $\gamma$  ligands. Naïve CD4<sup>+</sup>CD62L<sup>+</sup> T cells were incubated for five

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days in the presence of IL-6 and TGF $\beta$  and 0.01% DMSO. On day 5, a high level of intracellular cytokine expression is induced by combination of treatment with a phorbol ester, ionomycin, and brefeldin A, and T<sub>H</sub>-17 and T<sub>H</sub>1 cells identified by intracellular staining with antibodies to IL-17 and IFN- $\gamma$ , respectively. The percentage of IL-17 positive cells, or T<sub>H</sub>-17 cells, was calculated as a fraction of total live cells. OR-1050 decreases the proportion of IL-17<sup>+</sup> cells while OR-12872 increases the fraction of IL-17<sup>+</sup> cells. Each treatment was performed in duplicate (mean $\pm$ SD, n=2)

FIGS. 8A-D. An ROR $\gamma$  agonist (OR-12872) reverses antagonist (OR-1050) effects on regulation of T<sub>H</sub>-17 cell differentiation. The methods follow FIG. 7. Each treatment was performed in duplicate, and a representative scatterplot for each treatment is shown. FIG. 8A: control; FIG. 8B: 3  $\mu$ M OR-1050; FIG. 8C: 3  $\mu$ M OR-12872; FIG. 8D: 3  $\mu$ M OR-1050+3  $\mu$ M OR-12872.

FIG. 9A-D. OR-885 inhibits T<sub>H</sub>-17 differentiation and OR-12872 reverses its antagonistic effect. The methods follow FIGS. 7 and 8. Each treatment was performed in duplicate, and a representative scatterplot for each treatment is shown. FIG. 9A: control; FIG. 9B: 3  $\mu$ M OR-885; FIG. 9C: 1  $\mu$ M OR-112872; FIG. 9D: 3  $\mu$ M OR-885+1  $\mu$ M OR-12872.

FIG. 10. IL-17 release into culture medium was measured four days after induction of T<sub>H</sub>-17 differentiation in CD4<sup>+</sup> naïve murine T cells, following the methods of FIG. 7. Each antagonist (OR-885, OR-1050, OR-13571, and OR-2161) was incubated in the cultures at 3  $\mu$ M, in the presence or absence of OR-12872, also at 3  $\mu$ M. On day 4, culture supernatants were saved and IL-17 measured by ELISA.

FIGS. 11A-C. ROR $\gamma$  antagonist inhibits severity, weight loss, onset, Th-17 frequency and inflammation in EAE models. (FIGS. 11A-C) C57BL/6 mice were immunized with 150  $\mu$ g MOG<sub>35-55</sub> at day 0 to induce EAE and were dosed daily with corn oil (vehicle) or OR-1050 (50 mg/kg, 2 $\times$  per day) by oral gavage starting at day -1. At Day 26, the spinal cord was removed for analysis of inflammation (FIG. 11C).

FIGS. 11D-F SJL/J mice were immunized with 75  $\mu$ g PLP<sub>139-151</sub> (Day 0). Osmotic pumps delivering vehicle or OR-1050 (~30 mg/kg) were inserted at Days 3 and 4. Day of onset (FIG. 11E) compares the first day when EAE severity is 1 or higher. The ratio of Th-17 cells (CD4<sup>+</sup>CD8<sup>-</sup>IL17<sup>+</sup>) to total live cells was analyzed in a separate study (FIG. 11F) at day 9 after immunization with PLP where mice had been dosed daily with OR-1050 (100 mg/kg in HRC-6) since day -1. Statistics (panels A,B,D,F) were performed by Student's t-test. \*, P<0.05; #, P<0.01 (mean $\pm$ sem, n=7-10) or by the Mann-Whitney rank order test (FIGS. 11C, E, median values shown).

FIG. 11G shows the effect of OR-1050 on splenic T<sub>H</sub>-17 cells in C57BL/6 mice (29% inhibition, p<0.05, n=8-10) with no change in T<sub>H</sub>1 cells. C57BL/6 female mice were immunized with 150  $\mu$ g MOG<sub>33-55</sub> on study day 0. Mice were treated with either OR-1050 at 100 mg/kg or with vehicle (HRC-6) by oral gavage for 9 days starting day 0, Splenocytes were collected at Day 9 and stained for T<sub>H</sub>-17 (CD4<sup>+</sup>CD8<sup>-</sup>IL17<sup>+</sup>) and T<sub>H</sub>1 (CD4<sup>+</sup>CD8<sup>-</sup>IFN $\gamma$ <sup>+</sup>) cells. Statistics were performed by Student's t-test.

FIG. 12A. OR-1050 inhibits the onset of paralysis in a murine model of EAE. This is a separate statistical analysis of data presented in FIG. 11A. C57BL/6 mice were immunized with MOG<sub>35-55</sub>, an immunogenic peptide derived from myelin oligodendrocyte glycoprotein, to trigger symptoms of EAE. Mice were treated with corn oil vehicle or OR-1050 (15 mg/kg or 50 mg/kg) twice per day by gavage

starting one day before injection of MOG<sub>35-55</sub> and continuing for 25 days after injection. The day on which a visual, clinical score first reached 1 or greater was recorded for each mouse, and observations were carried out until day 26. By a rank order non-parametric test for multiple groups (Kruskal-Wallis, with Dunn's Multiple Comparison Test for significance), the onset of EAE (determined as a severity score of 1 or greater) in mice treated with 100 mg/kg OR-1050 per day is significantly ( $p < 0.05$ ) delayed compared to the vehicle control. A similar finding was obtained by analysis of day of onset determined by severity score of 2 or greater.

FIG. 12B. Bioavailability of OR-1050. OR-1050 was dosed in CD-1 mice at 5 mg/ml in HRC-6, a proprietary formulation that is commercially available from Pharmatek (San Diego, Calif.). Blood samples from triplicate animals were tested for levels of OR-1050 at multiple time points.

FIGS. 13A-F. IL-17 levels in lymph node cells from mice immunized with myelin-derived peptides respond to cognate peptide antigens, IL-23 and ROR $\gamma$  ligands. (FIG. 13A) SJL/J mice were immunized by complete Freund's adjuvant (CFA) only or by Proteolipid Protein residues 139-151 (PLP<sub>139-151</sub>) emulsified in CFA. Lymph nodes were cultured for 3 days in with or without 40  $\mu$ g/ml PLP in the presence or absence of OR-885. Culture media were analyzed by ELISA for IL-17. (FIGS. 13B-C) Lymph node cells from PLP-immunized SJL/J mice were cultured as before with the addition of 10 ng/ml IL-23 and 3  $\mu$ M compounds. At the end of 1 day or 3 days in culture, Th-17 (CD4<sup>+</sup>CD8<sup>-</sup>IL17<sup>+</sup>) cell frequency was measured by FACS (FIG. 13B) and compared to IL-17 levels in culture media (FIG. 13C). (FIG. 13D-F) Lymph node cells from MOG<sub>35-55</sub> immunized C57BL/6 mice were cultured for 3 days with 30  $\mu$ g/ml MOG and/or 10 ng/ml IL-23. ROR $\gamma$  ligands were not added (FIG. 13D), or added at the beginning of the culture at a single concentration (FIG. 13E, 1  $\mu$ M) or in multiple doses (FIG. 13F).

FIG. 14. Inhibition of IL-17 release in mouse lymph node cultures by the ROR $\gamma$  antagonist OR-885 can be reversed by the ROR $\gamma$  agonist OR-12872. The lymph node cells were culture in the presence of 10 ng/mL IL-23 and 40  $\mu$ g/mL PLP for three days under identical conditions to FIG. 13 and IL-17 in the culture supernatant was measured by ELISA.

FIGS. 15A-B. IL-22 release in lymph node cultures is regulated by ROR $\gamma$  ligands. Lymph node cells from MOG-immunized C57BL/6 mice were cultured for 3 days with 30  $\mu$ g/ml MOG, 10 mg/ml IL-23 (FIG. 15A) and 3  $\mu$ M compounds (FIG. 15B). Culture media were analyzed by IL-22 ELISA.

FIGS. 16A-C. Human IL-17 release from activated human T cells is inhibited by ROR $\gamma$  antagonist and is partially reversible in the presence of OR-12872, an ROR $\gamma$  agonist. In FIG. 16A, PBMCs were exposed to 1  $\mu$ g/ml Con A for 3 days and treated simultaneously with 3  $\mu$ M OR-885 or DMSO carrier in the presence or absence of 3  $\mu$ M OR-12872. In FIG. 16B, PBMCs were treated for 3 days in the presence 1  $\mu$ g/mL Con A and 3  $\mu$ M of the following compounds: OR-13571, OR-1050, or OR-12872 alone or in combination. In FIG. 16C, T cell blasts were treated with OR-1050, OR-13571, and OR-12872 in the presence of a phorbol ester for 18 hours to induce cytokine release.

FIGS. 17A-B. ROR $\gamma$  antagonist regulation of CIA in DBA/1 mice. Mice were immunized with bovine type II collagen (CII) at days 0 and 29. (FIG. 17A) Arthritic scores in hind limb (max score=8) for vehicle-treated (n=15) or 100 mg/kg/day OR-1050-treated (n=12) animals. (FIG. 17B) Disease intensity (Mean $\pm$ SEM) is the area under the curve (AUC) for hind limb clinical scores from day 24 to 48. AUC

scores for the two groups were compared by the Mann-Whitney rank order test (one-tailed) to determine statistical significance.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention includes the discovery that small molecule antagonists to retinoic acid related orphan receptor  $\gamma$  (ROR $\gamma$ ) inhibit T<sub>H</sub>-17 cell differentiation from naïve mouse T cells in cell culture, and inhibit the release of cytokines from mouse and human T<sub>H</sub>-17 cells. These small molecule antagonists have unique and beneficial properties for treatment of inflammatory and autoimmune diseases, since they inhibit the release of several pro-inflammatory cytokines from T<sub>H</sub>-17 cells, including IL-17 and IL-22. Conversely, a potent ROR $\gamma$  agonist can enhance T<sub>H</sub>-17 cell formation and reverse the antagonist effect. These small molecule inhibitors can be used to treat a variety of inflammatory and autoimmune disorders including, but not limited to, arthritis, diabetes, multiple sclerosis, uveitis, rheumatoid arthritis, psoriasis, asthma, bronchitis, allergic rhinitis, chronic obstructive pulmonary disease, atherosclerosis, *H. pylori* infections, ulcers resulting from *H. pylori* infections and inflammatory bowel disease (IBD). IBD includes, but is not limited to, Crohn's disease, ulcerative colitis and sprue.

ROR $\gamma$  agonists and antagonists are fairly specific to T<sub>H</sub>-17 cells and do not appear to have a primary effect on T<sub>H</sub>1 cells. Several strategies that inhibit development or activity of T<sub>H</sub>-17 cells for the treatment of MS and other autoimmune diseases can be envisioned, but these primarily involve treatment with macromolecules such as antibodies that block IL-23 or IL-17 (Bowman, Chackerian et al. 2006) or cytokines that inhibit T<sub>H</sub>-17 cell function (Batten, Li et al. 2006; Stumhofer, Laurence et al. 2006). ROR $\gamma$  antagonists offer the first approach to specifically block T<sub>H</sub>-17 development with small molecule drugs. An ROR $\gamma$  antagonist offers superior properties for human dosing and therapeutic use such as oral bioavailability. The following definitions are provided:

A receptor antagonist is a molecule that inhibits the normal physiological function of a receptor. Many drugs work by blocking the action of endogenous receptor agonists such as hormones and neurotransmitters. Antagonists that compete with an agonist for a receptor are competitive antagonists. Those that antagonize by other means are non-competitive antagonists.

A receptor agonist is a molecule that, in the nuclear receptor context, increases the transcriptional activity of a receptor or overcomes the activity of a competitive receptor antagonist.

A small molecule drug is an orally or intravenously bioavailable compound having a molecular weight less than about 600 daltons.

Related nuclear receptor. The human and mouse families of nuclear receptors comprise 48 members (Laudet 1999), and these have been arranged in both families (e.g., family 1, including both ROR $\gamma$  and LXR $\alpha$ ) and subfamilies (e.g., NR1F) that include ROR $\alpha$ , ROR $\beta$ , and ROR $\gamma$ . For the purposes of this discussion, related nuclear receptors are those that belong to a single receptor subfamily, such as NR1F, and unrelated nuclear receptors may belong to the same family but to different subfamilies.

A confirmed hit at ROR $\gamma$  is a small molecule compound that regulates transcription through ROR $\gamma$  and also regulates the affinity of the ROR $\gamma$  LBD with an appropriate coregulatory peptide in a validated assay for the receptor.

Potency. A measure of compound concentration required to activate or inhibit a pharmacological endpoint. Potency is often estimated as  $EC_{50}$  (effective concentration producing a 50% effect) or by similar measures known to those skilled in the art of pharmacology.

Rank order of potency is a method of comparing small molecule ligand pharmacology data from two separate assays. Compounds are ranked by  $EC_{50}$  or other measure of potency in each of the two assays. The purpose of this ranking is to provide a method of comparing the results of two different assays for which absolute compound potencies may differ.

A transcriptional assay for a nuclear receptor measures regulation of gene expression of a target gene that contains a response element for that receptor in its promoter region. Promoter regions can be engineered to contain appropriate response elements and these in turn can be coupled to a variety of reporter genes. Such assays are widely used to characterize nuclear receptor function and utilize target genes, such as chloramphenicol acetyl transferase (CAT), luciferase (LUC), and beta-lactamase (BLA). The activity of these enzymes is readily assayed in cell extracts or whole cells. Nuclear receptor assays also take advantage of the fact that the ligand-binding domain (LBD) of the receptor can function independently of its DNA-binding domain (DBD). Chimeric receptors that contain a common DBD, for example the DBD of the yeast transcriptional factor GAL4 (Webster, Green et al. 1988), are fused to the LBDs of individual nuclear receptors. Multiple nuclear receptor LBDs, fused to the same DBD, may be screened against a common target gene that contains the response element for that DBD (Schultz, Tu et al. 2000). Chimeric receptors are co-transfected individually with the common target gene. Ligands effects are determined after incubation for a sufficient period of time according to changes in level of expression of the target gene.

A biochemical assay for a nuclear receptor is carried out in the presence of partially purified receptor LBD and, directly or indirectly, measures binding of ligand. Two methods are commonly used: (i) competitive displacement of known, labeled ligand and (ii) recruitment of coregulatory peptide. Coregulatory peptide recruitment takes advantage of the fact that the receptor LBD will usually bind short peptides derived from conserved sequences within the so-called coregulatory proteins in a manner that depends on the presence or absence of ligand (Bramlett, Yao et al. 2000; Lee, Elwood et al. 2002; Wu, Chin et al. 2002). Coregulatory proteins in turn are required for transcriptional regulation in response to ligand binding to the receptor (McKenna and O'Malley 2002). Both receptor LBD and peptide are tagged with molecular markers, and the degree of association of these markers determined in the presence or absence of ligand.

ROR $\gamma$ . The gene that encodes ROR $\gamma$  (RORc) undergoes alternative splicing to give rise to two splicing isoforms, ROR $\gamma$  and ROR $\gamma$ t. The expression of ROR $\gamma$  is widespread, and mRNA for ROR $\gamma$  appears in liver, muscle, fat and many other tissues (Jetten, Kurebayashi et al. 2001). The ROR $\gamma$ t splicing isoform is predicted to generate a protein that is truncated by 21 amino acids at the N-terminus of ROR $\gamma$  (He, Defetos et al. 1998). ROR $\gamma$ t is expressed predominantly in thymocytes, lymph node precursors known as LTi cells, in T<sub>H</sub>-17 cells, and in other T cells, including a subpopulation of CD8<sup>+</sup>,  $\gamma\delta$ TCR<sup>+</sup>, and NKT cells (Ivanov, McKenzie et al. 2006; Ivanov and Littman. 2007). The ROR $\gamma$ t isoform

contains an LBD that is identical to ROR $\gamma$ . For the purpose of this application, the two receptors may be referred to collectively as ROR $\gamma$ .

ROR $\gamma$  Function. Nuclear receptors are thought to act primarily as nuclear transcription factors, by regulating gene expression. Some nuclear receptors have been shown to have acute, non-genomic effects that cannot be explained by gene transcription. Examples include the thyroid and estrogen receptors (Hiroi, Kim et al. 2006). Although a transcriptional assay is useful for identification of ROR $\gamma$  ligands, the ligand response of ROR $\gamma$  may include direct activation of other cell-signaling pathways, such as those mediated by kinases, phosphatases, or levels of intracellular messengers.

FITC—Flourescein isothiocyanate

APC—allophycocyanin

PE—phycoerythrin

LBD—ligand-binding domain

DBD—DNA-binding domain

FRET—Fluorescence resonance energy transfer

The T<sub>H</sub>-17 cell is recently-described subset of CD4<sup>+</sup> T helper cells that expresses IL-17 and IL-17F, as well as other cytokines, including pro-inflammatory cytokines such as IL-22 (Liang, Tan et al. 2006). The T<sub>H</sub>-17 cell also expresses the autocrine cytokine, IL-21. Other T cells may also express ROR $\gamma$ t and IL-17. The most prevalent type of IL-17<sup>+</sup> T cell in the gut is the TCR $\alpha\beta$ <sup>+</sup>CD4<sup>+</sup> T<sub>H</sub>-17 cell (Ivanov, McKenzie et al. 2006), while the  $\gamma\delta$  T cell is the primary source of IL-17 in response to pulmonary infection (Lockhart, Green et al. 2006). In addition, CD8<sup>+</sup> T cells,  $\gamma\delta$  T cells and NKT cells also express ROR $\gamma$ . Thus, T<sub>H</sub>-17 cells are a subset of IL-17<sup>+</sup> T cells. A significant fraction of the IL-17<sup>+</sup> T cells appear to be ROR $\gamma$ t<sup>+</sup> (Ivanov and Littman 2007).

Pharmaceutical composition refers to a mixture of ROR $\gamma$  antagonist or inhibitor, or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof, with other chemical components, such as diluents or carriers. The pharmaceutical composition facilitates administration of the compound to an organism. Multiple techniques of administering a compound exist in the art including, but not limited to, oral, injection, aerosol, parenteral, and topical administration. Pharmaceutical compositions can also be obtained by reacting compounds with inorganic or organic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

Carrier defines a chemical compound that facilitates the incorporation of a compound into cells or tissues. For example dimethyl sulfoxide (DMSO) is a commonly utilized carrier as it facilitates the uptake of many organic compounds into the cells or tissues of an organism.

Diluent defines chemical compounds diluted in water that will dissolve or suspend the compound of interest and preferably also stabilize the biologically active form of the compound. Salts dissolved in buffered solutions are utilized as diluents in the art. One commonly used buffered solution is phosphate buffered saline because it mimics the salt conditions of human blood. Since buffer salts can control the pH of a solution at low concentrations, a buffered diluent rarely modifies the biological activity of a compound.

Physiologically acceptable defines a carrier or diluent that is suitable for in vivo administration.

Pharmaceutically acceptable salt refers to a salt form of an original compound formed by association of a counterion with that compound, wherein the counterion is generally nontoxic. Pharmaceutical salts can be obtained, for example, by reacting a compound of the invention with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric

acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. Pharmaceutical salts can also be obtained by reacting a compound of the invention with a base to form a salt such as an ammonium salt, an alkali metal salt, such as a sodium or a potassium salt, an alkaline earth metal salt, such as a calcium or a magnesium salt, a salt of organic bases such as dicyclohexylamine, N-methyl-D-glutamine, tris(hydroxymethyl)methylamine, and salts with amino acids such as arginine, lysine, and the like.

Metabolite refers to a compound to which a ROR $\gamma$  antagonist or agonist is converted within the cells of a mammal. The pharmaceutical compositions of the present invention may include a metabolite of a ROR $\gamma$  antagonist instead of the ROR $\gamma$  antagonist. The scope of the methods of the present invention includes those instances where the ROR $\gamma$  antagonist is administered to the patient, yet the metabolite is the bioactive entity.

Prodrug refers to an agent that is converted into the parent drug in vivo. Prodrugs are often useful because, in some situations, they may be easier to administer than the parent drug. They may, for instance, be bioavailable by oral administration whereas the parent is not. The prodrug may also have improved solubility in pharmaceutical compositions over the parent drug. An example, without limitation, of a prodrug would be a compound of the present invention which is administered as an ester (the "prodrug") to facilitate transmittal across a cell membrane where water solubility is detrimental to mobility but which then is metabolically hydrolyzed to the carboxylic acid, the active entity, once inside the cell where water-solubility is beneficial. A further example of a prodrug might be a short peptide (polyamino-acid) bonded to an acid group where the peptide is metabolized to reveal the active moiety.

In a further aspect, the present invention relates to a method of treating a patient with a pharmaceutical composition as described herein.

The term "treating" or "treatment" does not necessarily mean total cure. Any alleviation of any undesired signs or symptoms of the disease to any extent or the slowing down of the progress of the disease can be considered treatment. Furthermore, treatment may include acts that may worsen the patient's overall feeling of well being or appearance. Treatment may also include lengthening the life of the patient, even if the symptoms are not alleviated, the disease conditions are not ameliorated, or the patient's overall feeling of well being is not improved.

#### ROR $\gamma$ Antagonists

The present invention provides compounds that selectively inhibit the transcriptional activity of the orphan nuclear receptor ROR $\gamma$ . These ROR $\gamma$  antagonists may be small organic molecules, proteins, peptides, nucleic acids, carbohydrates or antibodies. These compounds permit the properties of ROR $\gamma$  as a pharmacological target to be investigated in isolated cells and in animals. Further, the invention provides for novel methods of treating autoimmune disease and related conditions by inhibiting the function and/or activity of TH-17 cells, or by inhibiting the differentiation of TH-17 cells, or by inhibiting IL-17 and IL-22 release in a population of T cells that may include TH-17 cells (e.g., CD4<sup>+</sup> T cells) and other ROR $\gamma$ t+ IL-17+ T cells such as CD8<sup>+</sup> T cells, NKT, or TCR $\gamma$  $\delta$ + T cells, through suppression of the activity of ROR $\gamma$ . In examples shown herein, specific compounds that inhibit or activate transcription through the ROR $\gamma$  LBD, respectively ROR $\gamma$  antagonists or agonists, are described, and structurally distinct antagonists of ROR $\gamma$  transcription specifically inhibit

the differentiation of CD4<sup>+</sup> T<sub>H</sub>-17 cells from naïve CD4<sup>+</sup> T cells. Furthermore, the structurally distinct antagonists also inhibit the release of IL-17 from murine T<sub>H</sub>-17 cells during differentiation or from memory IL-17<sup>+</sup> T cells in lymph node cultures. In addition, the structurally diverse ROR $\gamma$  antagonists inhibit IL-17 release from human peripheral blood mononuclear cells (PBMCs).

The examples demonstrate that pharmacologically useful small molecules for cellular studies can be identified by receptor assays. To be useful, the molecules should be active in an assay of receptor-mediated transcription. Second, in addition to activity in a transcriptional assay, such molecules should be active in a second receptor assay that uses a mechanistically distinct readout, such as coactivator or coregulatory protein or peptide recruitment to receptor LBD as a function of ligand concentration (Heery, Kalkhoven et al. 1997; Lee, Elwood et al. 2002). The proximity of the ROR $\gamma$  LBD and the coregulatory peptide or macromolecule is measured by one of several well-established biophysical methods. Some of these methods, well known to practitioners of the art of studying protein-peptide interactions, include fluorescence resonance energy transfer (FRET), fluorescence polarization, time-resolved FRET (TR-FRET) with europium conjugates as donor, and Alpha Screen, in which laser-induced oxygen emission from a donor bead stimulates a fluorophore in an acceptor bead in close proximity (Iannone, Consler et al. 2001; Lee, Elwood et al. 2002; Xu, Stanley et al. 2002; Moore, Galicia et al. 2004; Li, Choi et al. 2005).

The biochemical assay excludes molecules that non-specifically regulate an ROR $\gamma$  reporter gene in culture. To allow definitive findings in cell experiments, a compound will ideally have an EC<sub>50</sub> < 1  $\mu$ M and measurable cytotoxicity only at higher concentrations. Finally, such molecules should be selective for ROR $\gamma$ . If this is not the case, a secondary control should be available, for example, an agonist that demonstrates the reversibility of antagonist effect. Without these controls, there is the opportunity to confuse an effect of a non-specific candidate ROR $\gamma$  ligand on T<sub>H</sub>-17 cells with an authentic activity of the small molecule mediated by interaction with the LBD of ROR $\gamma$ . The transcriptional and biochemical assays described herein can be used to determine the ability of any small molecule to act as an ROR $\gamma$  receptor antagonist or agonist.

The invention provides examples in which the validity of certain hits were further confirmed by demonstrating that a series of related compounds to the hit share a similar rank order of potency in both the transcriptional and biochemical assays over a wide range.

In view of the fact that ROR $\gamma$ t is required for CD4<sup>+</sup> T<sub>H</sub>-17 cell formation in mice (Littman and Eberl 2006), we tested whether ROR $\gamma$  ligands specifically inhibit T<sub>H</sub>-17 differentiation from naïve CD4<sup>+</sup> T cells in culture (Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006). T<sub>H</sub>-17 inhibition by small molecule modulation through ROR $\gamma$  was verified by several criteria. First, three separate structurally distinct ROR $\gamma$  antagonists (OR-1050, OR-885, and OR-13571, EC<sub>50</sub> < 1  $\mu$ M in the transcriptional assay) had comparable activity in T<sub>H</sub>-17 inhibition; second, antagonist effect could be reversed by a potent and specific agonist (OR-12872) to ROR $\gamma$ ; and third, the ROR $\gamma$  ligand effect was specific for cell culture differentiation of T<sub>H</sub>-17 but not T<sub>H</sub>-1 cells.

Further, the invention provides for molecules with a reasonable margin of safety. We demonstrated that one of the ROR $\gamma$  ligands, OR-1050, can be dosed in mice such that it should cause a pharmacological effect through ROR $\gamma$ . We



therefore examined the effect of the compound on function of the thymus, since the major pool of developing T lymphocytes, the double positive CD4<sup>+</sup>CD8<sup>+</sup> (DP) T cells, expresses ROR $\gamma$ t. Germline deletion of ROR $\gamma$  or ROR $\gamma$ t leads to an increased rate of DP thymocyte apoptosis and a reduction in DP thymocyte number to about 30% of control (Kurebayashi, Ueda et al. 2000; Sun, Unutmaz et al. 2000; Eberl and Littman 2004). Therefore, a model study with OR-1050 was used to investigate possible effects on thymic function. The ROR $\gamma$  antagonist OR-1050 also activates the nuclear receptors LXR $\alpha$  and LXR $\beta$ , and therefore the bioavailability of OR-1050 could be demonstrated by induction of triglyceride accumulation in liver since this is a well-defined endpoint for LXR ligands (Schultz, Tu et al. 2000; Beyer, Schmidt et al. 2004). The EC<sub>50</sub> for OR-1050 at LXR $\alpha$  (1.5  $\mu$ M) is higher than for ROR $\gamma$  (0.3  $\mu$ M) in transcriptional assays, suggesting that ROR $\gamma$  would also be antagonized in the drug-treated animals. In a six day study, liver triglycerides were markedly elevated by daily exposure to 100 mg/kg OR-1050, but the compound had no effect on the frequency or total number of the major cell types of the thymus. This invention therefore provides for compounds that will have limited effects on the function of the thymus.

Further, OR-1050 slows the onset of EAE in C57BL/6 female mice injected with a myelin-derived peptide. The data are consistent with the prediction that an ROR $\gamma$  antagonist free of LXR activity will be effective in this model. In addition, OR-1050 is demonstrated to have modest bioavailability. The invention provides a method for selecting compounds that have negligible or reduced LXR potency while maintaining or improving ROR $\gamma$  potency. T0901317 does not elevate liver triglyceride levels in mice where the LXR $\alpha$  and LXR $\beta$  receptors have been deleted (Schultz, Tu et al. 2000). More specific ROR $\gamma$  antagonists are predicted to have beneficial effects in animal models of CIA, EAE, and IBD while limiting the undesirable consequences of LXR activation, such as liver triglyceride accumulation and elevated serum LDL (Schultz, Tu et al. 2000; Beyer, Schmidt et al. 2004; Groot, Pearce et al. 2005), or other undesirable consequences, either for safety or efficacy, of activation, inhibition, or interaction with other known pharmacological targets.

In addition, the invention provides for compounds that suppress IL-17 expression in activated human T cells. Elevated IL-17 levels in disease tissue are strongly suggested to signal pathogenic involvement of T<sub>H</sub>-17 and other IL-17 positive cells.

The advantages of direct targeting of T<sub>H</sub>-17 cells with a small molecule drug could be very significant compared to the biologics that are very likely under development (Bowman, Chackerian et al. 2006). In one embodiment, a drug from the ROR $\gamma$  antagonist class has good oral bioavailability. In another embodiment, the drug has a half-life of four hours or more, enabling once or twice-daily administration. Nuclear receptor ligands have commonly given rise to orally bioavailable drugs; OR-1050 is an example of an orally bioavailable ROR $\gamma$  antagonist characterized in these studies. The advantage of an orally bioavailable drug is: (i) direct oral administration is feasible; (ii) unlike injectable biologics, which may have an extremely long half life, a small molecule drug with reasonable half-life can be withdrawn if necessary to limit side effects and (iii) small molecule drugs are readily manufactured. Such compounds may also be selected for clinical development by potency in animal models of EAE, CIA and IBD and by parallel in vivo studies of compound safety.

#### ROR $\gamma$ Agonists

ROR $\gamma$  agonists may be small organic molecules, proteins, peptides, nucleic acids, carbohydrates or antibodies. Particularly preferred are small organic molecules. The present invention also includes methods of increasing the number of T cells reactive to a specific antigen by administering an ROR $\gamma$  agonist in conjunction with, or subsequent to, administration of the antigen; and for enhancing the differentiation of T<sub>H</sub>-17 cells by contacting a population of T cells that may include T<sub>H</sub>-17 cells with an ROR $\gamma$  agonist. In one embodiment, agonists of ROR $\gamma$  are used to enhance or modulate an immune response. In another embodiment, an ROR $\gamma$  agonist is used to enhance the effectiveness of vaccine compositions. Vaccination remains the primary mechanism of inhibiting the spread of infectious agents. For newly discovered organisms, vaccination is the most highly favored option since drug therapy may require decades to provide clinical options. Although most healthy individuals respond to the foreign antigen presented by vaccination, there is a critical need to stimulate the immune response in the elderly, who are much less responsive to vaccination in the first place due to aging of their immune system. Furthermore, subunit vaccines, although easy to prepare since they are based on exogenously expressed proteins from the pathogen, are often not highly immunogenic. Thus, concomitant stimulation of the immune response will improve vaccine take. The vaccine compositions may be attenuated live vaccines, or non-replicating and/or subunit vaccines. In one embodiment, these vaccines induce memory T<sub>H</sub>-17 cells specific for the vaccine. Types of vaccines include, but are not limited to, tumor vaccines, viral vaccines (DNA, RNA or retroviral), bacterial vaccines and parasitic vaccines.

ROR $\gamma$  agonists can also be used to increase mucosal immunity to a preselected antigen by administering a mucosal immunity-enhancing amount of an ROR $\gamma$  agonist in conjunction with, or subsequent to, the antigen. The antigen can be bacterial, viral or a tumor antigen.

An ROR $\gamma$  agonist can be used to increase the immunogenicity of an immunogenic composition (e.g., vaccine composition) by administering the ROR $\gamma$  agonist in conjunction with, or subsequent to, the immunogenic composition. ROR $\gamma$  agonists can also be used to enhance induction, expression and/or release of a pro-inflammatory cytokine (e.g., IL-6, IL-17, IL-22, IL-3, TNF- $\alpha$ ), a pro-inflammatory cytokine receptor, a pro-inflammatory chemokine (e.g., CC chemokines, CXC chemokines, C chemokines, CX<sub>3</sub>C chemokines) or a pro-inflammatory chemokine receptor in cells capable of expressing the cytokine, cytokine receptor, chemokine or chemokine receptor, by administering the ROR $\gamma$  agonist to the cells. The administration may be in vitro, ex vivo or in vivo.

The ROR $\gamma$  agonists described herein also induce T<sub>H</sub>-17 cell differentiation and/or transcription of IL-17 and/or IL-22 in a population of T cells that may include T<sub>H</sub>-17 cells. The ROR $\gamma$  agonists may be administered in vitro, ex vivo or in vivo.

The pharmaceutical compositions described herein can be administered to a human patient per se, or in pharmaceutical compositions where they are mixed with other active ingredients, as in combination therapy, or suitable carriers or excipient(s). Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., 18th edition, 1990. The pharmaceutical compositions may also be administered to other mammals, including dogs, cats, sheep, pigs, horses, cows, and the like.

Thus, the veterinary use of these pharmaceutical compositions is also within the scope of the present invention.

Suitable routes of administration may, for example, include oral, rectal, topical, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intravenous, intramedullary injections, as well as intrathecal, direct intraventricular, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly in the renal or cardiac area, often in a depot or sustained release formulation. Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with a tissue-specific antibody. The liposomes will be targeted to and taken up selectively by the organ.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or tableting processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen. Any of the well-known techniques, carriers, and excipients may be used as suitable and as understood in the art; e.g., in Remington's Pharmaceutical Sciences, above.

For injection, the ROR $\gamma$  agonists/antagonists of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained by mixing one or more solid excipient with pharmaceutical combination of the invention, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

For topical administration, the compounds may be formulated for administration to the epidermis as ointments, gels, creams, pastes, salves, gels, creams or lotions, or as a transdermal patch. Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general

also containing one or more emulsifying agents, stabilizing agents, dispersing agents, suspending agents, thickening agents, or coloring agents.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally, including sublingually, which include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. A common cosolvent system used is the VPD co-solvent system, which is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant Polysorbate 80™, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of POLYSORBATE 80™; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g., polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

Many of the compounds used in the pharmaceutical combinations of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free acid or base forms.

Pharmaceutical compositions suitable for use in the present invention include compositions where the active ingredients are contained in an amount effective to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

The exact formulation, route of administration and dosage for the pharmaceutical compositions of the present invention can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl et al. 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p. 1). Typically, the dose range of the composition administered to the patient can be from about 0.5 to 1000 mg/kg of the patient's

body weight. The dosage may be a single one or a series of two or more given in the course of one or more days, as is needed by the patient. Note that for almost all of the specific compounds mentioned in the present disclosure, human dosages for treatment of at least some condition have been established. Thus, in most instances, the present invention will use those same dosages, or dosages that are between about 0.1% and 500%, more preferably between about 25% and 250% of the established human dosage. Where no human dosage is established, as will be the case for newly-discovered pharmaceutical compounds, a suitable human dosage can be inferred from ED<sub>50</sub> or ID<sub>50</sub> values, or other appropriate values derived from in vitro or in vivo studies, as qualified by toxicity studies and efficacy studies in animals.

Although the exact dosage will be determined on a drug-by-drug basis, in most cases, some generalizations regarding the dosage can be made. The daily dosage regimen for an adult human patient may be, for example, an oral dose of between 0.1 mg and 6000 mg of each ingredient, preferably between 1 mg and 5000 mg, e.g. 25 to 5000 mg or an intravenous, subcutaneous, or intramuscular dose of each ingredient between 0.01 mg and 100 mg, preferably between 0.1 mg and 60 mg, e.g. 1 to 40 mg of each ingredient of the pharmaceutical compositions of the present invention or a pharmaceutically acceptable salt thereof calculated as the free base, the composition being administered 1 to 4 times per day. Alternatively the compositions of the invention may be administered by continuous intravenous infusion, preferably at a dose of each ingredient up to 400 mg per day. Thus, the total daily dosage by oral administration of each ingredient will typically be in the range 1 to 2500 mg and the total daily dosage by parenteral administration will typically be in the range 0.1 to 400 mg. Suitably the compounds will be administered for a period of continuous therapy, for example for a week or more, or for months or years.

In one embodiment, the dose of the pharmaceutical composition comprising a ROR $\gamma$  agonist or antagonist or a pharmaceutically acceptable salt, prodrug, derivative or metabolite thereof, is from about 10 to about 50 mg per day.

In another embodiment, the ROR $\gamma$  antagonist is administered daily without resulting in weight loss or hypertriglyceridemia.

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compositions should be administered using a regimen that maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may

for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the drug for human or veterinary administration. Such notice, for example, may be the labeling approved by the U.S. Food and Drug Administration for prescription drugs, or the approved product insert. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

The compositions described herein may also be used in the preparation of a medicament for treatment of any of the disorders described above.

#### Example 1

This example describes ligands to ROR $\gamma$  and confirms their receptor regulation properties in two mechanistically distinct receptor assays. Transcriptional assays for mouse and human ROR $\gamma$  (mROR $\gamma$  and hROR $\gamma$ ) were implemented in cultured cells. A biochemical assay, using exogenously expressed mROR $\gamma$  LBD, was established to measure ligand-mediated regulation of coregulatory peptide recruitment in vitro. The transcriptional assay measures the product of a reporter gene, such as an enzyme, whose expression is in turn regulated by the ROR $\gamma$  LBD. The biochemical assay responds to a ligand-dependent conformational change in the receptor LBD that changes receptor affinity for coregulatory peptide. The ligands to ROR $\gamma$  described here were predominantly identified in the transcriptional assay and confirmed in the biochemical assay.

This example also describes several additional tests that were commonly performed to confirm the authenticity of a hit to ROR $\gamma$ . These tests also verified the validity of the transcriptional and biochemical assays used. In all cases, the activity of a compound was also tested in transcriptional assays for the orphan nuclear receptors ROR $\alpha$ , ROR $\beta$ , and SF-1 to demonstrate specificity and to rule out a non-specific effect on the assay system as an explanation for the apparent activity. In some cases, close analogs of a confirmed hit were obtained by synthesis or purchase from a commercial source. The series of compounds related to the confirmed hit was then tested in the biochemical and transcriptional assays. The rank order of the potency of the compounds was then compared. If closely similar for the two assays, the findings demonstrated that minor chemical modifications had equivalent effects on compound interaction with the ROR $\gamma$  LBD in each assay. The data imply in turn that the compounds are binding to the same binding pocket. Even though absolute EC<sub>50</sub> values may differ in a rank order comparison, the observation that relative values are similar provides strong confirmation of the conclusion that the identified compounds act directly through ROR $\gamma$  and not by a secondary mechanism that is assay but not receptor-specific. This results presented in this example demonstrate that the transcriptional and biochemical assays provide an internally consistent method of characterizing ROR $\gamma$  ligand potency and that more potent compounds can be identified using this assay technology by a program of additional screening or directed medicinal chemistry synthesis.

#### Methods

Transcriptional Assay. Gal4-mROR $\gamma$  was generated by inserting the LBD of mouse ROR $\gamma$  (Ile-251-Lys-516) into the EcoRI-HindIII site of pFA-CMV. The vector pFA-CMV (Stratagene, La Jolla, Calif.) contains the yeast Gal4 DNA binding domain (amino acids 1-148) upstream of the multiple cloning site where the EcoRI and HindIII restriction enzyme sites are found. The fragment of mROR $\gamma$  was a PCR product using the template of pM-mROR $\gamma$  (Medvedev, Yan et al. 1996). The complete Gal4-mROR $\gamma$  nucleotide sequence is shown below:

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15 (Gal4 DBD) (SEQ ID NO: 7)
ATGAAGCTACTGTCTTCTATCGAACAAGCATGCGATATTTGCCGACTT
AAAAGCTCAAGTGCTCCAAAGAAAAACCGAAGTGCGCCAAGTGTCTG
AAGAACAACCTGGGAGTGTGCTACTCTCCCAAACCAAAGGTCTCCG
20 CTGACTAGGGCACATCTGACAGAAGTGAATCAAGGCTAGAAAGACTG
GAACAGCTATTTCTACTGATTTTTCTCGAGAAGACCTTGACATGATT
TTGAAAATGGATTCTTTACAGGATATAAAAGCATTGTTAACAGGATTA
25 TTTGTACAAGATAATGTGAATAAAGATGCCGTACAGATAGATTGGCT
TCAGTGGAGACTGATATGCCTCTAACATTGAGACAGCATAGAATAAGT
GCGACATCATCATCGGAAGAGAGTAGTAACAAAGGTCAAAGACAGTTG
30 ACTGTATCGCCG
(linker) (SEQ ID NO: 8)
GGATCCGCCCGGGCTGGAATTCGC
35 (mROR $\gamma$  LBD) (SEQ ID NO: 9)
ATTCCCAGTTTCTGCAGTGCCCCAGAGGTACCATATGCCTCTCTGAC
AGACATAGAGTACCTGGTACAGAATGTCTGCAAGTCCTTCCGAGAGA
40 CATGCCAGCTGCGACTGGAGGACCTTCTACGGCAGCGCACCAACCTC
TTTTACGGGAGGAGGTGACCAGCTACCAGAGGAAGTCAATGTGGGA
GATGTGGGAGCGCTGTGCCACCACCTCACTGAGGCCATTGATGATG
45 TGGTGGAGTTTGCAAGCGGCTTTCAGGCTTCATGGAGCTCTGCCAG
AATGACCAGATCATACTACTGACAGCAGGAGCAATGGAAGTCGTCTT
AGTCAGAATGTGCAGGGCTACAATGCCAACAACCACACAGTCTTTT
50 TTGAAGGCAAATACGGTGGTGTGGAGCTGTTTCGAGCCTTGGGCTGC
AGCGAGCTCATCAGCTCCATATTTGACTTTTCCACTTCTCAGCGC
CCTGTGTTTTTCTGAGGATGAGATTGCCCTCTACACGGCCCTGGTTC
TCATCAATGCCAACCGTCTGGGCTCCAAGAGAAGAGGAGAGTGGAA
55 CATCTGCAATACAATTTGGAAGTGGCTTTCATCATCATCTCTGCAA
GACTCATCGACAAGGCCCTCCTAGCCAAGCTGCCACCCAAAGGAAAAC
TCCGGAGCCTGTGCAGCCAACATGTGAAAAGCTGCAGATCTTCCAG
60 CACCTCCACCCCATCGTGGTCCAAGCCGCTTCCNCCACTCTATAA
GGAACTCTTCAGCACTGATGTTGAATCCCCTGAGGGGCTGTCAAAGT
GA
65

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The complete Gal4-mROR $\gamma$  protein sequence is shown below:

(Gal4 DBD) (SEQ ID NO: 10)  
 MKLLSSIEQACDICRLKLLKCSKEKPKCAKCLKNNWECRYSPKTK  
 RSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMSLQDIK  
 ALLTGLFVQDNVNKDAVTDRLASVETDMPLTLRQHRISATSSSEE  
 SSNKGQRQLTVSP  
 (linker) (SEQ ID NO: 11)  
 GSARAGIR  
 (mROR $\gamma$  LBD) (SEQ ID NO: 12)  
 IPSFCSAPEVPYASLTDIEYLVQNVCKSFRETQCQLRLEDLLRQRT  
 NLFSREEVTSYQRKSMWEMWERCAHHLTEAIQYVVEFAKRLSGFM  
 ELCQNDQIILLTAGAMEVVLVRMCRAYNANNHTVFFEGKYGGVEL  
 FRALGCSELISSIFDFSHFLSALCFSEDEIALYALVLINANRPG  
 LQEKRRVEHLQYNLELAFHHHLCKTHRQGLLAKLPPKGLRSLCS  
 QHVEKLQIFQHLHPIVVQAAFPLYKELFSTDVESPEGLSK

Gal4-hROR $\gamma$  was generated by inserting the ligand-binding domain of human ROR $\gamma$  LBD (Ser-253-Lys-518) into pFA-CMV by BamHI-KpnI sites. The vector pFA-CMV (Stratagene, La Jolla, Calif.) contains the yeast Gal4 DNA binding domain (amino acids 1-148) at N-terminal of the multiple cloning sites. The fragment of hROR $\gamma$  LBD was a PCR product using the template of a commercial clone from Invitrogen (Clone ID: 5186655; Vector: pCMV-SPORT6). The complete Gal4-hROR $\gamma$  nucleotide sequence is shown below:

(Gal4 DBD) (SEQ ID NO: 7)  
 ATGAAGCTACTGTCTTCTATCGAACAAGCATGCGATATTTGCCGAC  
 TTA AAAAGCTCAAGTGCTCCAAAGAAAACCGAAGTGCGCCAAGTG  
 TCTGAAGAACAACCTGGGAGTGTGCTACTCTCCAAAACCAAAGG  
 TCTCCGCTGACTAGGGCACATCTGACAGAAGTGAATCAAGGCTAG  
 AAAGACTGGAACAGCTATTTCTACTGATTTTTCTCGAGAAGACCT  
 TGACATGATTTTTGAAAATGGATTCTTTACAGGATATAAAAGCATTG  
 TTAACAGGATTATTTGTACAAGATAATGTGAATAAAGATGCCGTCA  
 CAGATAGATTGGCTTCAGTGGAGACTGATATGCCTCTAACATTGAG  
 ACAGCATAGAATAAGTGGCAGATCATCATCGGAAGAGAGTAGTAAC  
 AAAGGTCAAAGACAGTTGACTGTATCGCCG  
 (linker) (SEQ ID NO: 13)  
 GGATCC  
 (hROR $\gamma$  LBD) (SEQ ID NO: 14)  
 AGCCCCAGTTTCCGCAGCACACCGGAGGCACCCTATGCCTCCCTGA  
 CAGAGATAGAGCACCTGGTGCAGAGCGTCTGCAAGTCTACAGGGA  
 GACATGCCAGCTGCGGCTGGAGGACCTGCTGCGGCAGCGCTCCAAC  
 ATCTTCTCCCGGAGGAAGTGACTGGCTACCAGAGGAAGTCCATGT  
 GGGAGATGTGGGAACGGTGTGCCACCCACCTACCGAGGCCATTCA

-continued

GTACGTGGTGGAGTTCGCCAAGAGGCTCTCAGGCTTTATGGAGCTC  
 TGCCAGAATGACCAGATTGTGCTTCTCAAAGCAGGAGCAATGGAA  
 5 TGGTGC TGGTTAGGATGTGCCGGGCTACAATGCTGACAACCGCAC  
 GGTCTTTTTTGAAGGCAAATACGGTGGCATGGAGCTGTTCCGAGCC  
 TTGGGCTGCAGCGAGCTCATCAGCTCCATCTTTGACTTCTCCACT  
 10 CCCTAAGTGCCTTGCACTTTTCCGAGGATGAGATTGCCCTCTACAC  
 AGCCCTTGTCTCATCAATGCCCATCGGCCAGGGCTCCAAGAGAAA  
 AGGAAAGTAGAACAGCTGCAGTACAATCTGGAGCTGGCCTTTCATC  
 15 ATCATCTCTGCAAGACTCATCGCCAAAGCATCCTGGCAAAGCTGCC  
 ACCCAAGGGGAAGCTTCGGAGCCTGTGTAGCCAGCATGTGGAAAGG  
 CTGCAGATCTTCCAGCACCTCCACCCCATCGTGGTCCAAGCCGCTT  
 20 TCCCTCCACTCTACAAGGAGCTCTTCCAGCACTGAAACCGAGTCACC  
 TGTGGGGCTGTCCAAGTGA

The complete Gal4-hROR $\gamma$  protein sequence is shown below:

(Gal4 DBD) (SEQ ID NO: 10)  
 MKLLSSIEQACDICRLKLLKCSKEKPKCAKCLKNNWECRYSPK  
 30 TKRSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMSL  
 QDIKALLTGLFVQDNVNKDAVTDRLASVETDMPLTLRQHRISA  
 TSSSEESSNKGQRQLTVSP  
 (linker) (SEQ ID NO: 15)  
 GS  
 (hROR $\gamma$  LBD) (SEQ ID NO: 16)  
 40 SPSFRSTPEAPYASLTDIEYLVQSVCKSYRETQCQLRLEDLLR  
 QRSNIFSREEVTGYQRKSMWEMWERCAHHLTEAIQYVVEFAK  
 RLSGFMELCQNDQIVLLKAGAMEVVLVRMCRAYNADNRTVFF  
 45 EGKYGMELFRALGCSELISSIFDFSHLSALHFSEDEIALY  
 TALVLINAHRPGLQEKRKVEQLQYNLELAFHHHLCKTHRQSI  
 LAKLPPKGLRSLCSQHVERLQIFQHLHPIVVQAAFPLYKE  
 LFTETESPVGLSK

50 Four other orphan nuclear receptors were similarly cloned as Gal4-LBD hybrids in pFA-CMV by PCR as follows for initial screening studies: human SF-1 (NR5A1, aa 198-462) at BamHI and XbaI restriction enzymes sites; mROR $\alpha$  (NR1F1, aa 266-523) at XmaI-HindIII restriction enzymes sites; human ROR $\beta$  (NR1F2, aa 201-459) at EcoRI and KpnI restriction enzyme sites; and mouse LRH-1 (NR5A2, aa 311-561) at EcoRI and HindIII restriction enzyme sites. A firefly luciferase reporter gene for Gal4 DBD hybrid 60 receptors, such as Gal4-ROR $\gamma$ , contains five copies of the Gal4 17-mer response element in its promoter and the coding sequence for firefly luciferase (pG5-luc, Promega). CHO (Chinese Hamster Ovary) cells were cultured in Ham's F12 medium supplemented with 10% fetal calf serum (Gemini Biologicals), 10  $\mu$ g/ml penicillin and streptomycin, 65 and were maintained in a humidified 37 $^{\circ}$  C. incubator (Thermo Electron, Steri-Cycle) with 5% CO $_2$ . 24 hours

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before transfection, cells were plated in T175 flasks ( $5 \times 10^6$  cells/flask) or T75 flasks ( $1.7 \times 10^6$  cells/flask). Transient transfection of CHO cells was performed using TransIT-CHO reagents (Minis, Madison, Wis.) with a mixing ratio of 2:6:1 between plasmid DNA (in  $\mu\text{g}$ ), TransIT-CHO (in p1) and CHO-mojo (in  $\mu\text{l}$ ). As an example, 9  $\mu\text{g}$  pG5-luc, 8.75  $\mu\text{g}$  pcDNA3 and 0.25  $\mu\text{g}$  receptor plasmid were transfected into each T175 flask.

Four hours after transfection, cells were dislodged with trypsin and seeded into a 384-well plate at 8,000 cells/well using a Titertek Multidrop 384. Edge effects within the 384-well plate were minimized by leaving plates at room temperature for 30 min so that cells plated evenly within each well (Lundholt, Scudder et al. 2003). Plates are maintained at  $37^\circ\text{C}$ . in a humidified atmosphere with 7%  $\text{CO}_2$  overnight. Four (4) hours after cell seeding, compounds dissolved in DMSO at 10 mM were added to cells at final concentrations of 20 micromolar ( $\mu\text{M}$ ) to 20 nanomolar (nM) in 0.2% DMSO in triplicate plates. Approximately 40 hours later, cytotoxicity in the assay well was characterized by addition of the dye resazurin (O'Brien, Wilson et al. 2000) to a final concentration of 3  $\mu\text{M}$ . After incubation for 2 hours at 37 degrees centigrade, the conversion of resazurin to resorufin was measured by fluorescence (excitation at 570 nm and emission at 615 nm). Living cells catalyze dye conversion while cells that lack metabolic activity do not. Percent viability was determined as 2 hour fluorescence, minus background at  $t=0$  hours, normalized to DMSO controls. For detection of luciferase activity, media was removed from plates after the measurement of resazurin conversion, and SteadyLite luciferase reagent (Perkin-Elmer) was added (30  $\mu\text{l}$ /well). Luminescence is detected with a Victor 2-V plate reader (Perkin-Elmer) and normalized to luciferase activity of DMSO control wells alone.

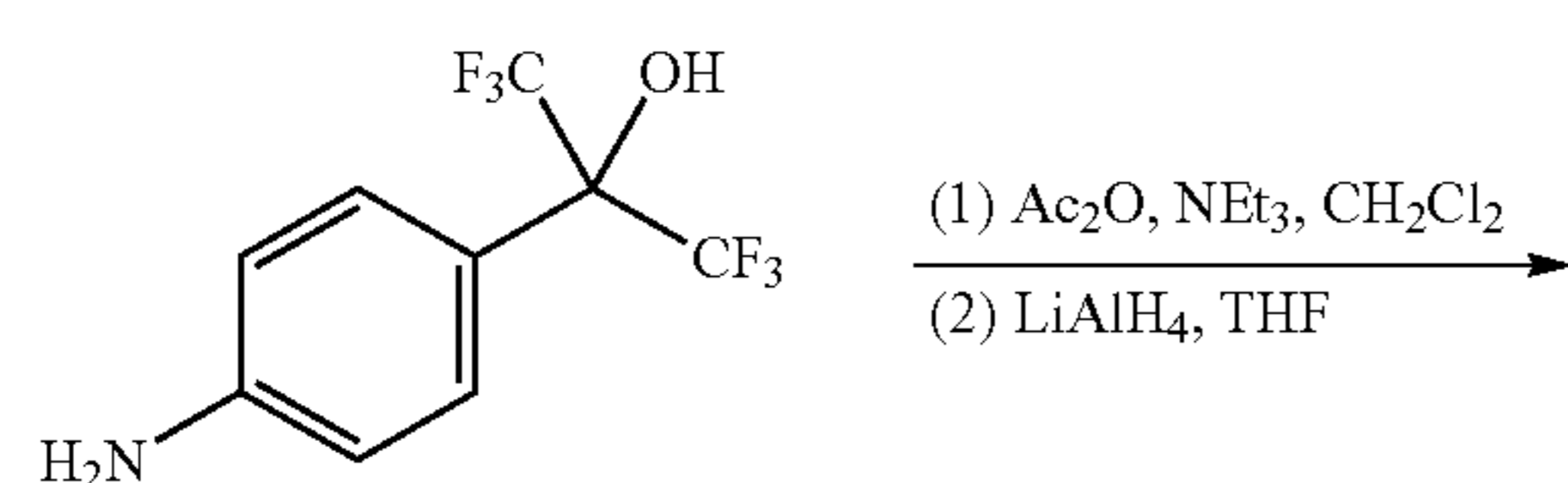
Compounds Described. The following test compounds were obtained commercially: T0901317 (Cayman Chemicals); rockogenin or OR-885 (Steraloids, Newport, R.I.);  $5\alpha$ ,  $20\alpha$ ,  $22\alpha$ ,  $25\text{D}$ -Spirostan- $3\beta$ ,  $12\beta$ -diol-11-one or OR-345 (Steraloids); hyodeoxycholic acid methyl ester or OR-942 and hyodeoxycyclic acid or OR-412 (Steraloids); 11-oxo ursolic acid acetate or OR-13571 (Microsource, Gaylordsville, Conn.) and AG-205/33159060 (Specs, Delft, The Netherlands) or OR-2161; OR-133008 (Specs AG-690/40752395); OR-133097 (Specs AG-690/40698971); OR-133099 (Specs AG-690/40699006); OR-133167 (Specs AG-690/40752726); and OR-133171 (Specs AG-690/40752859).

#### Compounds Synthesized.

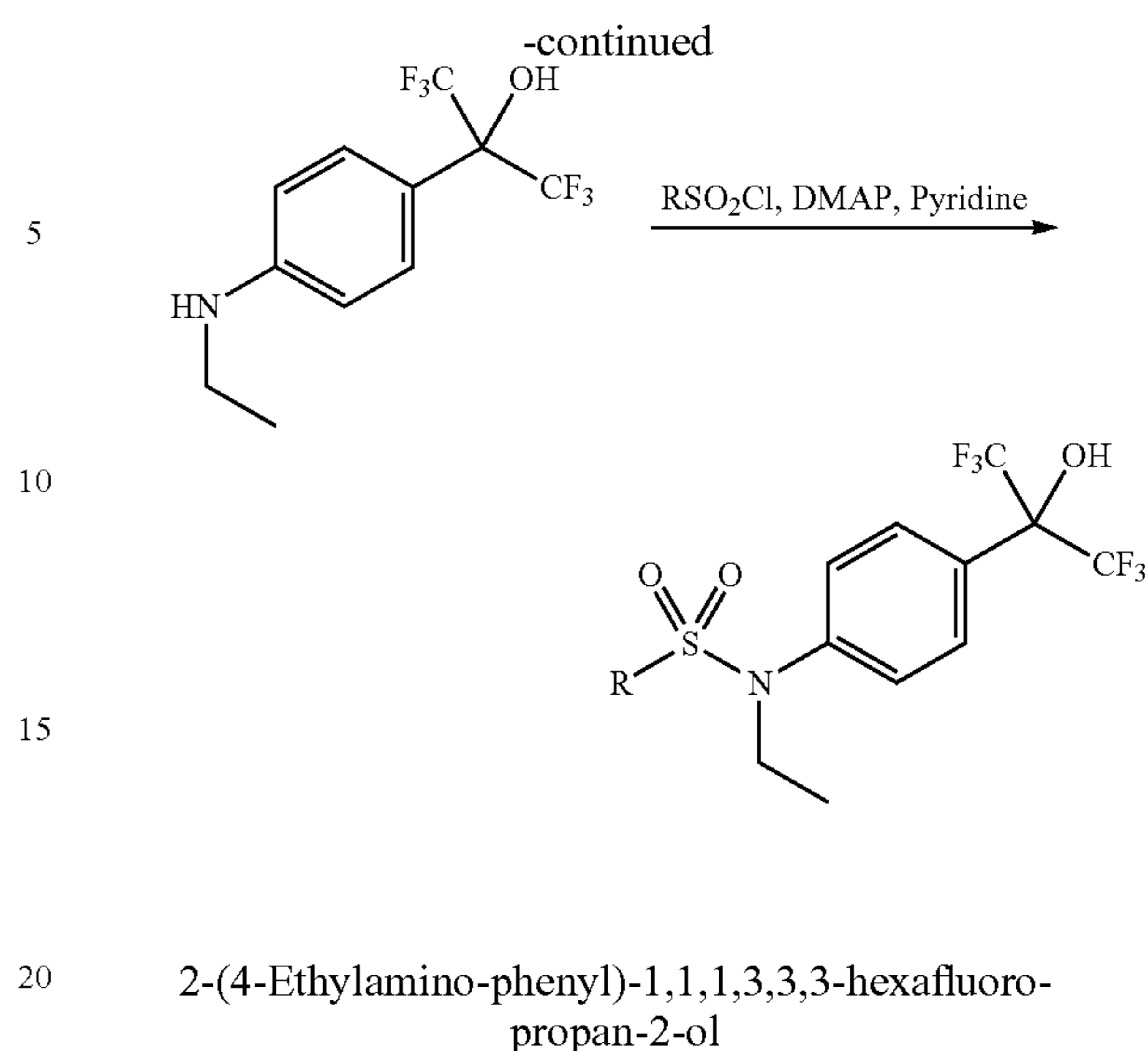
The following describes synthesis of a representative antagonist (e.g., OR-1050) and a representative agonist (e.g., OR-12872) to  $\text{ROR}\gamma$ . All starting materials were commercially available. Related antagonists and agonists, where not purchased commercially, were synthesized by analogous methods.

Abbreviations:  $\text{Ac}_2\text{O}$ —acetic anhydride;  $\text{NEt}_3$ —triethylamine; THF—tetrahydrofuran; DMAP—4-Dimethylaminopyridine; EDCI—(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride; TBSCl—tert-butyl-chloro-dimethylsilane; DMF—dimethylformamide; TBAF—Tetra-n-butylammonium fluoride.

Synthetic Scheme for OR-1050 where R=Para-nitrophenyl:



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To a solution of 3.0 gm of 2-(4-Amino-phenyl)-1,1,1,3,3,3-hexafluoro-propan-2-ol (Matrix Scientific, Columbia, S.C.) in 30 ml of methylene chloride was added 2.4 ml of triethyl amine, 1.2 ml of acetic anhydride, and 100 mg of dimethylaminopyridine. The mixture was allowed to stir at room temperature for 16 hrs. An additional 2.4 ml of triethyl amine and 1.2 ml of acetic anhydride was added and the mixture was stirred for an additional 6 hrs. The solution was evaporated and purified by column chromatography (1:1 Ethyl Acetate:Hexane) to give 3.1 gm of material that was used in the next step. To this material dissolved in 30 ml of tetrahydrofuran cooled to 0 degrees C. was added 1.6 gm of lithium aluminum hydride. This solution was stirred for 16 hrs and quenched with 1 N sodium hydroxide. This was then extracted with ethyl acetate and dried over anhydrous sodium sulfate. Chromatography gave 2.188 gm of the named product.  $^1\text{H}$  NMR was consistent with the structure.

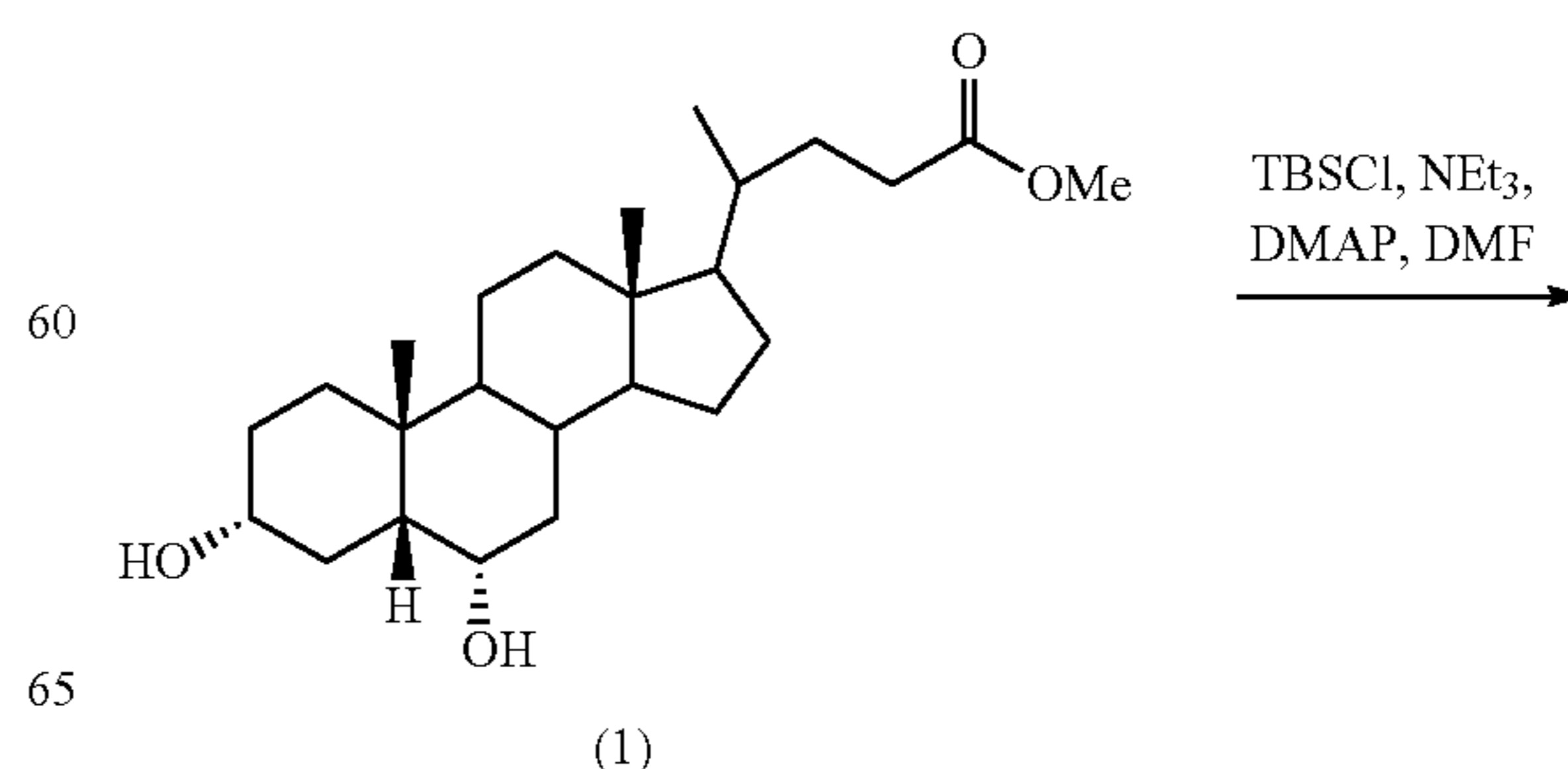
N-Ethyl-4-nitro-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide (OR-1050)

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To a solution of 104 mg 2-(4-Ethylamino-phenyl)-1,1,1,3,3,3-hexafluoro-propan-2-ol in 3 ml of pyridine was added 104 mg 4-Nitro-benzenesulfonyl chloride and 10 mg of dimethylaminopyridine. The mixture was stirred for 16 hrs and then concentrated in vacuo. After chromatography (1:3 Ethyl Acetate:Hexane), 129 mg of the named product was obtained.  $^1\text{H}$  NMR was consistent with the structure.

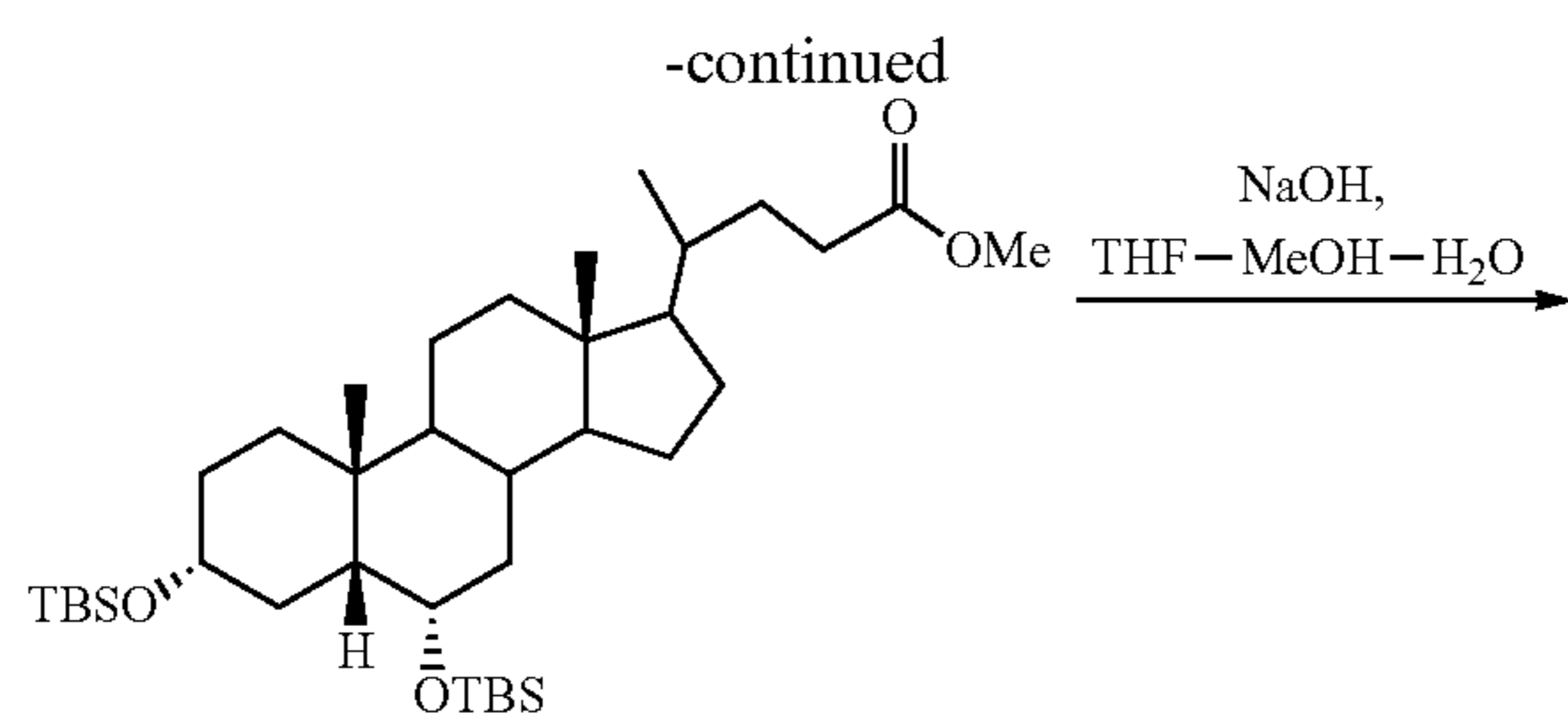
Synthetic Scheme for OR-12872

55

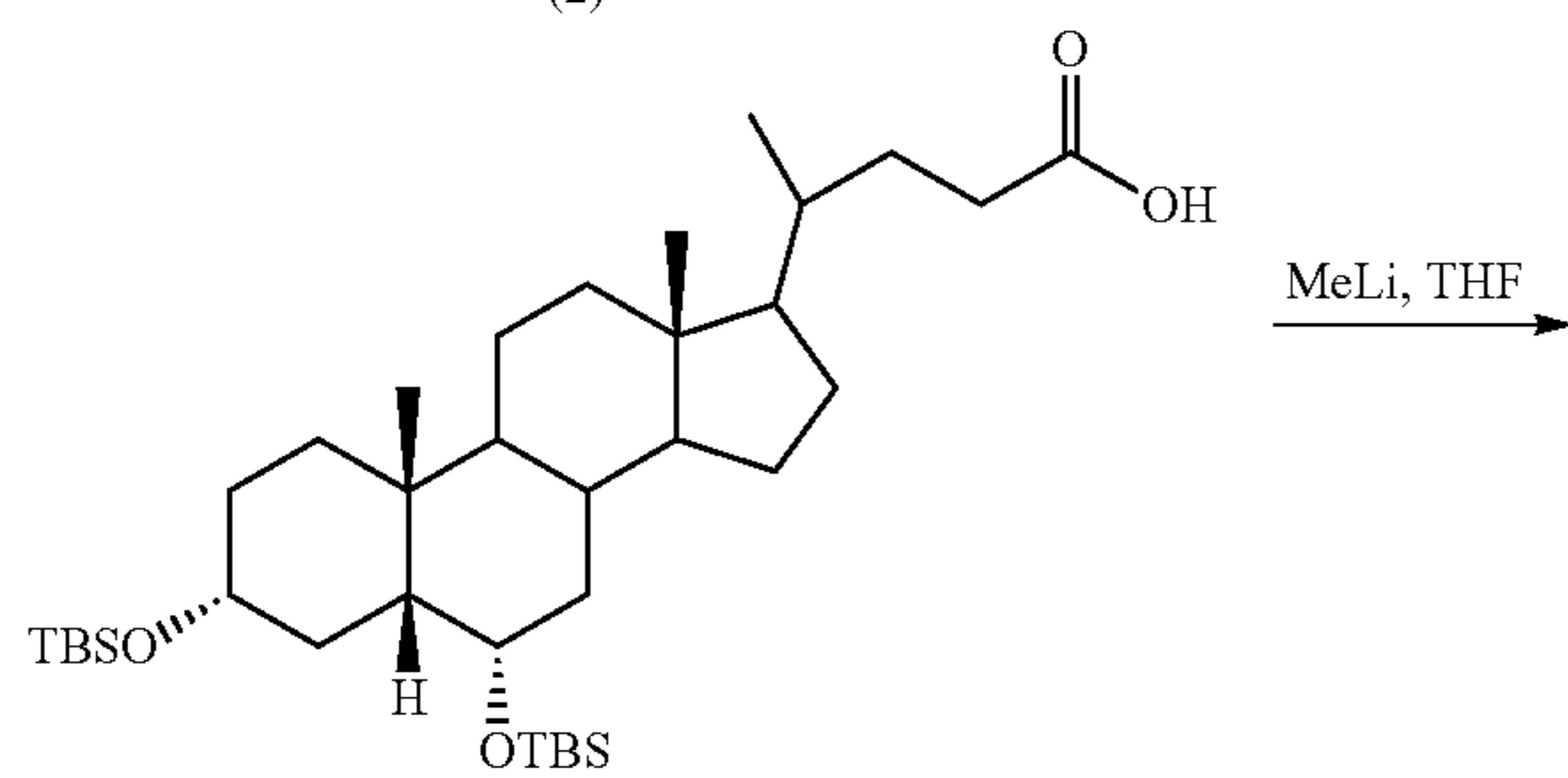


65

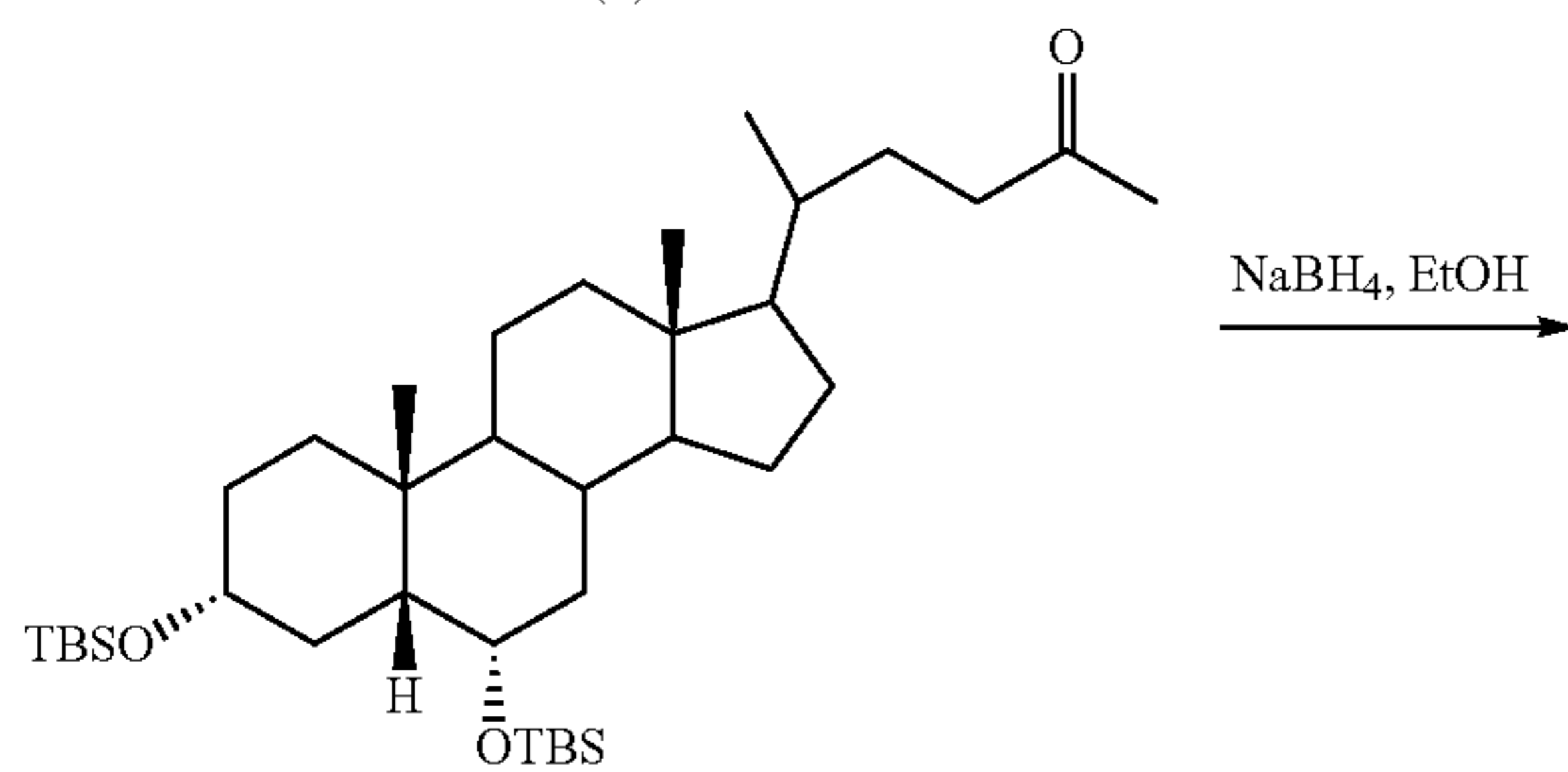
31



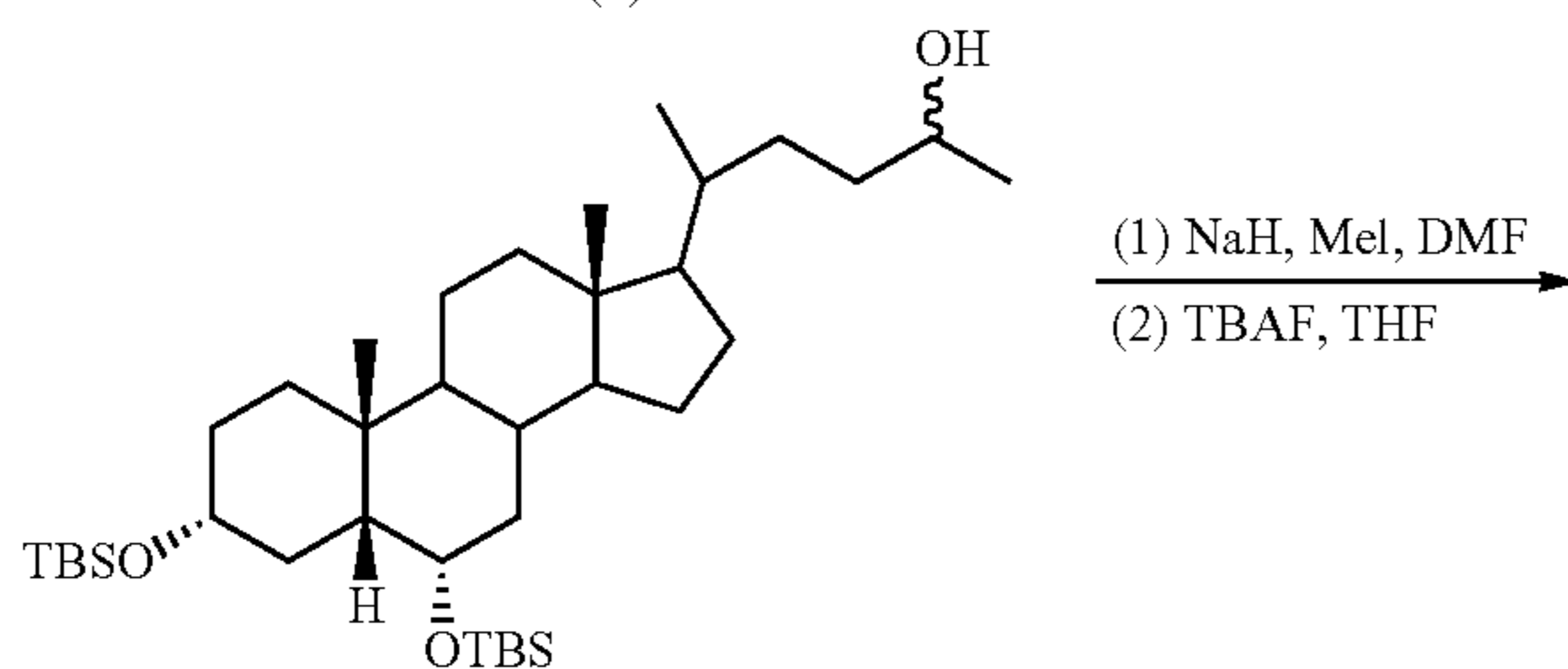
(2)



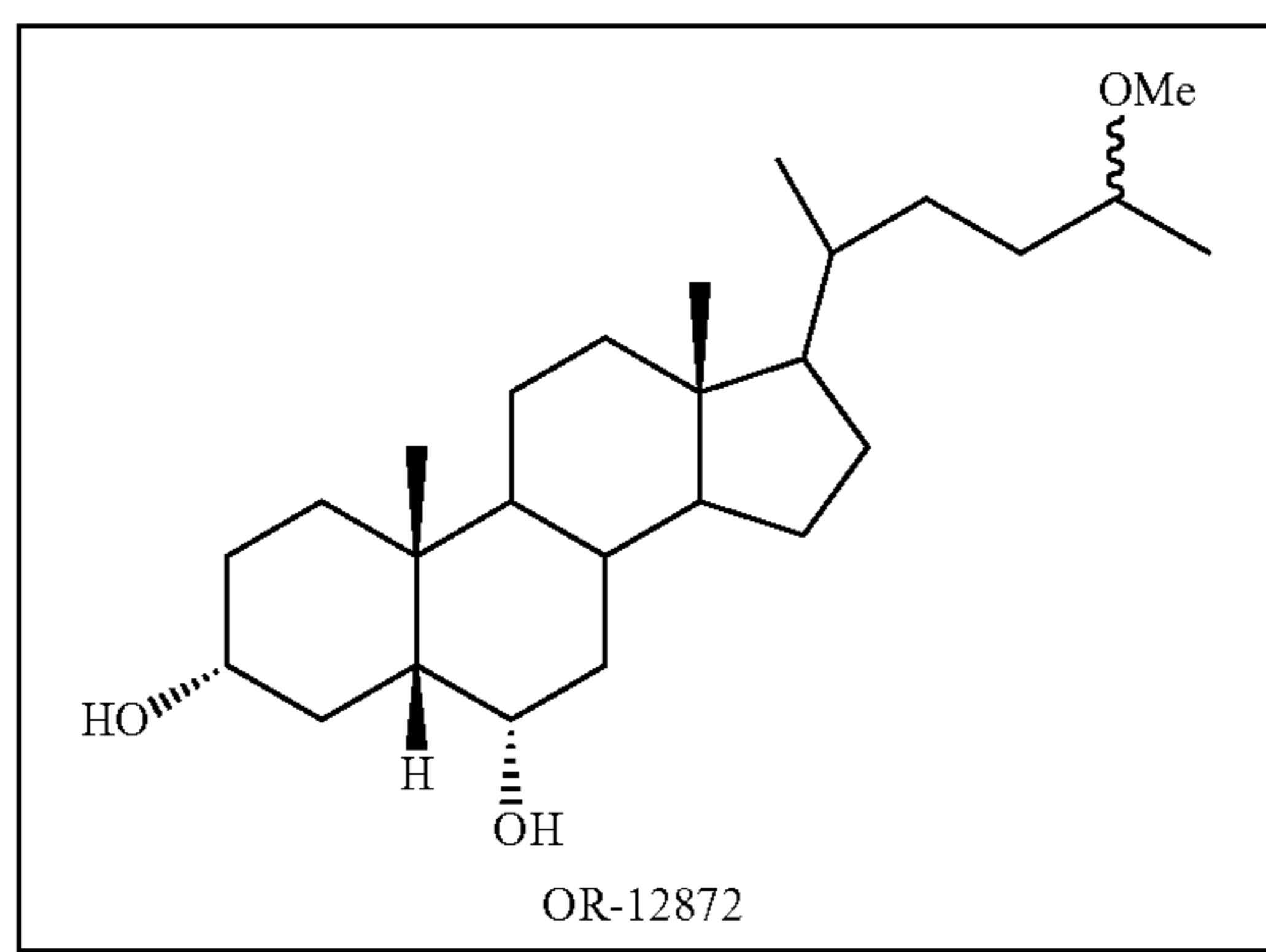
(3)



(4)



(5)



4R-[3R,6R-Bis-(tert-butyl-dimethyl-silanyloxy)-10R,13R-dimethyl-5R-8S-9S-14S-hexadecahydro-cyclopenta[a]phenanthren-17R-yl]-pentanoic acid methyl ester (2)

To a solution of 8.974 gm of hydoxycholeic acid methyl ester (4R-(3R,6R-Dihydroxy-10R,13R-dimethyl-5R-8S-9S-

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14S-hexadecahydro-cyclopenta[a]phenanthren-17R-yl)-pentanoic acid methyl ester) (1) (Steraloids, Newport, R.I.) in 60 ml of dimethyl formamide was added 12 ml of triethylamine, 8.3 gm of tert-Butyl-chloro-dimethyl-silane and 270 mg of dimethyl-aminopyridine. The mixture was stirred at room temperature for 16 hrs and concentrated in vacuo. After chromatography (1:9 Ethyl Acetate:Hexane), 13.748 gm of the named product (2) was obtained. <sup>1</sup>H NMR was consistent with the structure.

4R-[3R,6R-Bis-(tert-butyl-dimethyl-silanyloxy)-10R,13R-dimethyl-5R-8S-9S-14S hexadecahydro-cyclopenta[a]phenanthren-17R-yl]-pentanoic acid (3)

To a solution of 4-[3,6-Bis-(tert-butyl-dimethyl-silanyloxy)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthren-17-yl]-pentanoic acid methyl ester (9.41 gm) in 15 ml of tetrahydrofuran, 10 ml of methanol and 10 ml of water was added 620 mg of sodium hydroxide. The mixture was then stirred at room temperature for 16 hrs and concentrated in vacuo. After acidification the residue was extracted with ethyl acetate, dried over anhydrous sodium sulfate, filtered and concentrated in vacuo. Chromatography (1:3 Ethyl Acetate:Hexane) gave 8.6 gm of the title compound (3). <sup>1</sup>H NMR was consistent with the structure.

5R-[3R,6R-Bis-(tert-butyl-dimethyl-silanyloxy)-10R,13R-dimethyl-5R-8S-9S-14S-hexadecahydro-cyclopenta[a]phenanthren-17R-yl]-hexan-2-one (4)

To a solution of 4-[3,6-Bis-(tert-butyl-dimethyl-silanyloxy)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthren-17-yl]-pentanoic acid (3.419 gm) in 30 ml of tetrahydrofuran at 0 degrees C. was added 7.6 ml of a 1.6 M solution of methyl lithium. The mixture was stirred at 0 degrees C. for 30 min and quenched with 20 ml water followed by 20 ml of 1N hydrochloric acid. The mixture was extracted with ethyl acetate, dried over anhydrous sodium sulfate and concentrated in vacuo. Chromatography (1:9 Ethyl Acetate:Hexane) gave 0.985 gm of the title compound (4). <sup>1</sup>H NMR was consistent with the structure.

5R-[3R,6R-Bis-(tert-butyl-dimethyl-silanyloxy)-10R,13R-dimethyl-5R-8S-9S-14S-hexadecahydro-cyclopenta[a]phenanthren-17R-yl]-hexan-2-ol (5)

To a solution of 516 mg of 5-[3,6-Bis-(tert-butyl-dimethyl-silanyloxy)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthren-17-yl]-hexan-2-one in 10 ml of ethanol was added 250 mg of sodium borohydride. The mixture was stirred for 1 hr and concentrated in vacuo, 20 ml of water was added followed by 30 ml of ethyl acetate and 10 ml of 1N hydrochloric acid. The mixture was extracted with ethyl acetate, dried over anhydrous sodium sulfate and concentrated in vacuo. Chromatography (1:9 Ethyl Acetate:Hexane) gave 512 mg of the title compound (5). <sup>1</sup>H NMR was consistent with the structure.

17R-(4-Methoxy-1R-methyl-pentyl)-10R,13R-dimethyl-5R-8S-9S-14S-hexadecahydro-cyclopenta[a]phenanthrene-3R,6R-diol (OR-12872)

To a solution of 262 mg of (5) in 6 ml of dimethyl formamide was added 110 ml of methyl iodide followed by 29 mg of a 60% oil dispersion of sodium hydride. The mixture was stirred for 16 hr and concentrated in vacuo. The

mixture was extracted with ethyl acetate, dried over anhydrous sodium sulfate and concentrated in vacuo. Chromatography (1:9 Ethyl Acetate:Hexane) gave 257 mg of an intermediate that was dissolved in 10 ml of tetrahydrofuran and 2.4 ml of a 1M tetrahydrofuran solution of tetra-

butylammonium fluoride. The mixture was then stirred at room temperature for 16 hrs and then at 60 C. for 2 hrs. After concentration in vacuo and chromatography (1:9 Ethyl Acetate:Hexane), 154 mg of the title compound (OR-12872) was produced. <sup>1</sup>H NMR was consistent with the structure.

Compound Nomenclature. Compounds have the following IUPAC names: OR-1048, 4-Bromo-N-ethyl-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; OR-1052, N-Ethyl-4-methyl-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; OR-1050, N-Ethyl-4-nitro-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; OR-1047, 4-Butyl-N-ethyl-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; OR-1031, N-Ethyl-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; T0901317, N-(2,2,2-Trifluoro-ethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; OR-1030, N-Ethyl-N-[4-(2,2,2-trifluoro-1-methoxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; OR-1046, {Benzenesulfonyl-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-amino}-acetic acid.

OR-12872, 17-(4-Methoxy-1-methyl-pentyl)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthrene-3,6-diol; OR-12866, 17-(4-Methoxy-1,4-dimethyl-pentyl)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthrene-3,6-diol; OR-942, 4-(3,6-Dihydroxy-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthren-17-yl)-pentanoic acid methyl ester; OR-12863, 17-(4-Hydroxy-1-methyl-butyl)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthrene-3,6-diol; OR-12870, 5-(3,6-Dihydroxy-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthren-17-yl)-hexan-2-one; OR-12868, 4-(3,6-Dihydroxy-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthren-17-yl)-pentanoic acid dimethylamide; OR-12864, 17-(4-Methoxy-1-methyl-butyl)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthrene-3,6-diol; OR-12865, 17-(4-Hydroxy-1,4-dimethyl-pentyl)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthrene-3,6-diol; OR-12871, 17-(4-Hydroxy-1-methyl-pentyl)-10,13-dimethyl-hexadecahydro-cyclopenta[a]phenanthrene-3,6-diol; OR-412, 4-(3,6-Dihydroxy-hexadecahydro-cyclopenta[a]phenanthren-17-yl)-pentanoic acid; OR-12867, 4-(3,6-Dihydroxy-hexadecahydro-cyclopenta[a]phenanthren-17-yl)-pentanoic acid methylamide; OR-12869, 4-(3,6-Dihydroxy-hexadecahydro-cyclopenta[a]phenanthren-17-yl)-1-piperidin-1-yl-pentan-1-one;

OR-13571 (11-oxo-ursolic acid acetate), 10-Acetoxy-1,2,6a,6b,9,9,12a-heptamethyl-13-oxo-1,3,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-octadecahydro-2H-picene-4a-carboxylic acid.

The following descriptions are available for two of the natural compounds tested: OR-885 (CAS registry number, 16653-52-4, Rockogenin, 5a, 20 $\alpha$ , 22 $\alpha$ , 25D-SPIROSTAN-3 $\beta$ , 12 $\beta$ -DIOL, Steraloids catalogue number S0300-000) and OR-345 (5 $\alpha$ , 20 $\alpha$ , 22 $\alpha$ , 25D-SPIROSTAN-3 $\beta$ , 12 $\beta$ -DIOL-11-ONE, Steraloids catalogue number S0400-000).

OR-2161 is N-(4,6-Di-piperidin-1-yl-[1,3,5]triazin-2-yl)-N'-(2-methoxy-3-nitro-benzylidene)-hydrazine (Specs catalog number AG-205/33159060); OR-133008 is 2-{5-[(4-Chloro-phenylamino)-methyl]-4-ethyl-4H-[1,2,4]triazol-3-

yl sulfanyl}-N-naphthalen-1-yl-acetamide (Specs AG-690/40752395); OR-133097 is 2-{5-[(3-Chloro-phenylamino)-methyl]-4-ethyl-4H-[1,2,4]triazol-3-ylsulfanyl}-N-naphthalen-1-yl-acetamide (Specs AG-690/40698971); OR-133099 is 2-{4-Ethyl-5-[(3-trifluoromethyl-phenylamino)-methyl]-4H-[1,2,4]triazol-3-ylsulfanyl}-N-naphthalen-1-yl-acetamide (Specs AG-690/40699006); OR-133167 is 2-{5-[(3-Chloro-phenylamino)-methyl]-4-methyl-4H-[1,2,4]triazol-3-ylsulfanyl}-N-naphthalen-1-yl-acetamide (Specs AG-690/40752726); OR-133171 is 2-{5-[(3-Chloro-4-methyl-phenylamino)-methyl]-4-ethyl-4H-[1,2,4]triazol-3-ylsulfanyl}-N-naphthalen-1-yl-acetamide (Specs AG-690/40752859).

Biochemical Assay. GST-ROR $\gamma$  was generated by subcloning the ROR $\gamma$ -LBD into pET-41a, a vector which expresses GST fusion proteins in bacteria. A biochemical assay developed with GST-ROR $\gamma$  purified from bacteria had a poor response to ligand. The receptor was expressed in a baculovirus expression vector (Luckow and Summers 1989) in insect S19 cells. Expression in S19 cells, as an alternative to bacterial expression, was also successfully used to generate ROR $\alpha$  LBD in a form suitable for protein crystallization (Kallen, Schlaeppli et al. 2002).

The cloning steps for GST-ROR $\gamma$  were as follows. The mROR $\gamma$  LBD was excised from Gal4-mROR $\gamma$  (see above for cloning steps) at BamHI and HindIII sites of Sequence 1 and cloned into the corresponding sites of pET-41a(+) (EMD Biosciences, San Diego, Calif.). A GST-mROR $\gamma$  fragment was then excised from pET-41a(+) at XbaI and XhoI sites and cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, Calif.) restricted at NheI and XhoI sites. Plasmid GST-mROR $\gamma$ -pcDNA3.1 was used as a PCR template to generate a GST-mROR $\gamma$  fragment that was later cloned into pENTR/D-TOPO vector (Invitrogen, Carlsbad, Calif.) by standard cloning techniques recommended by the manufacturer of the vector. The nucleotide sequence of GST-mROR $\gamma$  is shown below:

```
(GST)
(SEQ ID NO: 17)
ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTT
CAACCCACTCGACTTCTTTTGGAAATATCTTGAAGAAAAATAT
GAAGAGCATTGTATGAGCGCGATGAAGGTGATAAATGGCGA
AACAAAAGTTTGAATGGGTTTGGAGTTTCCCAATCTTCTCT
TATTATATTGATGGTGATGTTAAATTAACACAGTCTATGGCC
ATCATACGTTATATAGCTGACAAGCACACATGTTGGGTGGT
TGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCG
GTTTTGGATATTAGATACGGTGTTCGAGAATTGCATATAGT
AAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTA
CCTGAAATGCTGAAAATGTTCTGAAGATCGTTTATGTCATAAA
ACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATG
TTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATG
TGCCTGGATGCGTTCCCAAATTAGTTTTGTTTTAAAAAACGT
ATTGAAGCTATCCCAAAATTGATAAGTACTTGAAATCCAGC
AAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTT
GGTGGTGGCGACCATCCTCCAAAATCGGAT
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-continued

(linker) (SEQ ID NO: 18)  
 GGTTCAACTAGTGGTTCTGGTCATCACCATCACCATCACTCC  
 GCGGGTCTGGTGCCACGCGGTAGTACTGCAATTGGTATGAAA  
 GAAACCGCTGCTGCTAAATTCGAACGCCAGCACCTGGACAGC  
 CCAGATCTGGGTACCGGTGGTGGCTCCGGTGATGACGACGAC  
 AAGAGTCCATGGGATATCGGGATCCGCCCGGGCTGGAATT  
 CGC  
 (mROR $\gamma$  LBD) (SEQ ID NO: 9)  
 ATTCCAGTTTCTGCAGTGCCCCAGAGGTACCATATGCCTCT  
 CTGACAGACATAGAGTACCTGGTACAGAATGTCTGCAAGTCC  
 TTCCGAGAGACATGCCAGCTGCGACTGGAGGACCTTCTACGG  
 CAGCGCACCAACCTCTTTTCACGGGAGGAGGTGACCAGCTAC  
 CAGAGGAAGTCAATGTGGGAGATGTGGGAGCGCTGTGCCAC  
 CACCTCACTGAGGCCATTAGTATGTGGTGGAGTTTGCCAAG  
 CGGCTTTCAGGCTTCATGGAGCTCTGCCAGAATGACCAGATC  
 ATACTACTGACAGCAGGAGCAATGGAAGTCGTCTAGTCAGA  
 ATGTGCAGGGCTACAATGCCAACAACACACAGTCTTTTTT  
 GAAGCAAATACGGTGGTGTGGAGCTGTTTCGAGCCTTGGGC  
 TGCAGCGAGCTCATCAGCTCCATATTTGACTTTTCCCACTTC  
 CTCAGCGCCCTGTGTTTTTCTGAGGATGAGATTGCCCTCTAC  
 ACGGCCCTGGTTCTCATCAATGCCAACCGTCCTGGGCTCCAA  
 GAGAAGAGGAGAGTGAACATCTGCAATACAATTTGAACTG  
 GCTTTCATCATCATCTCTGCAAGACTCATCGACAAGGCCTC  
 CTAGCCAAGCTGCCACCCAAAGGAAAACCTCCGGAGCCTGTGC  
 AGCCAACATGTGGAAAAGCTGCAGATCTTCCAGCACCTCCAC  
 CCCATCGTGGTCCAAGCCGCCTTCCNCCACTCTATAAGGAA  
 CTCTTCAGCACTGATGTTGAATCCCTGAGGGGCTGTCAAAG  
 TGA

The protein sequence of GST-mROR $\gamma$  is shown below:

(Gal4 DBD) (SEQ ID NO: 10)  
 MKLLSSIEQACDLCRLKLLKCSKEKPKCAKCLKNWECRYSPK  
 TKRSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSL  
 QDIKALLTGLFVQDNVNDKDAVTDRLASVETDMPPLTLRQHRISA  
 TSSSEESSNKGQRQLTVSP  
 (linker) (SEQ ID NO: 11)  
 GSARAGIR  
 (mROR $\gamma$  LBD) (SEQ ID NO: 12)  
 IPSFCSAPEVPYASLTDI EYLVQNVCKSFRETQCLRLEDLLRQ  
 RTNLF SREEVTSYQRKSMWEMWERCAHHLTEAIQYVVEFAKRL

-continued

SGFMELCQNDQI ILLTAGAMEVVLVRMCRAYNANNHTVFFEGK  
 YGGVELFRALGCSELISSIFDFSHFLSALCFSEDEIALYALV  
 5 LINANRPGLQEKRRVEHLQYNLELAFHHHLCKTHRQGLLAKLP  
 PKGKLRSLCSQHVEKIQIFQHLHPIVVQAAFXYKELFSTDV  
 ESPEGLSK

10 For baculoviral expression of ROR $\gamma$ , the cDNA fragment of GST-ROR $\gamma$ -LBD, was subcloned from pET-41a into the pENTR/D-TOPO vector using a commercially-available kit (BaculoDirect, Invitrogen). Briefly, GST-ROR $\gamma$  carried by pENTR/D-TOPO was transfected into mono-layer Sf9 cells (8 $\times$ 10<sup>5</sup>/well in 6-well plates) by Cellfectin (Invitrogen). Sf9 cells were incubated at 27° C. in a non-humidified incubator for 72 hours. Cell media was collected as P1 viral stock and used to further infect Sf9 cells to generate P2 and P3 virus. Significant signs of infection were observed during generation of P3 virus. A plaque assay was performed to determine the titer of P3 virus and to isolate single plaques. P4 virus was generated from a single plaque and propagated on a larger scale.

Adherent SF9 cells were infected with baculovirus carrying GST-ROR $\gamma$  and were cultured for 72 hours. Sf9 cell were then subject to a brief sonication in a lysis buffer (20 mM phosphate, pH 6.8, 50 mM KCl, 50 mM NaCl, 0.5 mM EDTA, 50% glycerol, 0.001% NP-40 and 1 mM DTT) and centrifuged at 3000 rpm for 15 minutes. GST-ROR $\gamma$  protein represents an estimated 20-30% of total soluble protein in the cytosolic supernatant as determined by SDS-PAGE and immunoblot with anti-GST antibody (Sigma). Some protein remained in an insoluble fraction after centrifugation. We found that addition of ROR $\gamma$  ligands (preferably 5  $\mu$ M T0901317) in the culture media increased the fraction of GST-ROR $\gamma$  in the cytosolic fraction. Cleared cell lysate was stored at -70° C. and is directly utilized in the coregulatory peptide recruitment assay.

To carry out the biochemical assay, receptor, peptides, and fluorescently-labeled probes to GST and biotin were incubated under these conditions: purified biotinylated peptide K (0.2  $\mu$ M), 80 nM anti-GST coupled to allophycocyanin (APC), 25 nM streptavidin-R-phycoerythrin (SA-RPE), that specifically binds the biotinylated peptide, 20 mM sodium phosphate, pH 6.8, 50 mM KCl, 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, 0.001% NP-40, and 1 mM DTT were incubated with the Sf9 cell lysate diluted in the range of 1:10 to 1:50, following methods from the literature (Coward, Lee et al. 2001; Drake, Zhang et al. 2002; Lee, Elwood et al. 2002), and incubated at room temperature for 2 hours. The labeled probes anti-GST-APC and SA-RPE were purchased from Prozyme (San Leandro, Calif.). Compounds were added directly from a DMSO solution to a final concentration of 1-2% DMSO. Fluorescence was measured on a Wallac Victor 2 V plate reader equipped with a 670/40 nm filter (Omega Optical, Brattleboro, Vt.) for APC emission (Channel A) and a 600/25 bandpass filter for monitoring the RPE emission (Channel B). The FRET signal is calculated as: (the difference between the ratio of Channel B/Channel A in the presence of an active peptide (such as peptide K) and the Channel A/Channel B ratio in the presence of the corresponding inactive peptide (peptide Kmut)) $\times$ 100 or in the complete absence of peptide.

#### Results

65 Members of the ROR $\alpha$ , ROR $\beta$ , and ROR $\gamma$  family of orphan nuclear receptors are generally recognized as having a high basal activity in cell culture systems (Jetten, Kure-

bayashi et al. 2001). The transcriptional activity of Gal4-mROR $\gamma$  in CHO cells and in the choriocarcinoma cell line JEG-3 was >20-fold higher than a mutated form of Gal4-mROR $\gamma$  (E502Q or E/Q). The E/Q mutation inactivates a highly conserved residue of the helix 12 region of the LBD that is required for recruitment of transcriptional coactivators in most well-characterized nuclear receptors (Li, Lambert et al. 2003), and is predicted to abrogate transcription from the ROR $\gamma$  LBD. An assay for detecting ROR $\gamma$  ligands was developed in CHO cells, and about 100,000 individual small molecule compounds from various commercial sources tested in this assay in several formats (e.g., in 96 or 384-well plates, or following 24-hour or 40-hour incubation with test compounds). Two classes of ROR $\gamma$  antagonists are shown below. Structure 1 represents a genus of non-steroidal antagonists in which R1=H, C1-C6 Alkyl, F, Cl, Br, I, NO<sub>2</sub>; R2=C1-C4 Alkyl; and X=OH. Structure 3 represents a genus of steroidal antagonists in which X=O or is absent (X=H,H). Any antagonist encompassed by these generic structures is within the scope of the present invention. The ability of any such compound, or any other compound, to act as an ROR $\gamma$  antagonist may be easily determined using the assays described herein. Structure 2 represents a genus of steroidal agonists in which X=C(E)(F)(G); wherein E and F are independently selected from H, lower alkyl or E and F taken together form a carbonyl group; G is selected from OH, O-lower alkyl or N(lower alkyl)<sub>2</sub>. Any agonist encompassed by these generic structures is within the scope of the present invention. The ability of any such compound, or any other compound, to act as an ROR $\gamma$  agonist may be easily determined using the assays described herein.

FIG. 3 shows that two compounds, T0901317 and OR-1050 (Table 1), both inhibit the high basal transcriptional activity of ROR $\gamma$ . In separate studies, we determined that the two compounds had no effects on the transcription of ROR $\alpha$ , ROR $\beta$  or SF-1 as GAL4 hybrids in the same format as the GAL4-ROR $\gamma$  assay. Further, T0901317 <10  $\mu$ M was not cytotoxic to CHO cells while OR-1050 was not cytotoxic at 50  $\mu$ M. All antagonists and agonists identified in this example were similarly specific for ROR $\gamma$  in the transcriptional assay and were non-cytotoxic to CHO cells at 20  $\mu$ M or, if not, were non-cytotoxic at a concentration 10-fold greater than the EC<sub>50</sub> in the transcriptional assay.

Because the basal transactivation activity of GST-ROR $\gamma$  is high, other compounds that bind ROR $\gamma$  and have only a modest effect on transcription when tested alone in cell culture may be overlooked. Some of these compounds could be classified as ROR $\gamma$  agonists. These may activate, rather than repress, ROR $\gamma$ -mediated processes in cells or animals. Accordingly, a screening assay for ROR $\gamma$  agonists was carried out in which one of the ROR $\gamma$  antagonists identified, T0901317, was added to suppress the basal transcriptional activity of GAL4-ROR $\gamma$ . FIG. 4A demonstrates a method for the identification of ROR $\gamma$  agonists. Compounds that only modestly elevated transcription in the absence of T0901317 (for example, the steroidal antagonist OR-942, see Table 2), but induced a much greater elevation in the presence of 1-3  $\mu$ M T0901317, determined as the ratio between wells containing OR-942 and those lacking OR-942, were further investigated in dose response assays (FIG. 4A).

To further confirm the properties of ROR $\gamma$  agonists and antagonists, a biochemical assay for ROR $\gamma$  was developed. This assay can be used to screen for agonists or antagonists of ROR $\alpha$ , ROR $\gamma$  or ROR $\beta$ . This assay is based on contacting a compound with a labeled, expressed ROR LBD and a peptide that includes residues 710-720 (RTVLQLLGNP; SEQ ID NO: 2) of human RIP140, and measuring the

proximity of the two labels, wherein binding of the labeled peptide identifies the compound as an antagonist and displacement of the labeled peptide identifies the compound as an agonist.

In FIG. 4B, the addition of OR-942 causes the recruitment of peptide K (ERRTVLQLLGNPTK; SEQ ID NO: 4), a 15-mer identical to amino acid residues 708-722 of transcriptional coregulatory protein human RIP140 (Iannone, Consler et al. 2001), to the ROR $\gamma$  LBD and increases the FRET signal. In the presence of OR-942, T0901317 depresses the FRET signal. Further, T0901317 causes a rightward shift in the dose-response curve for OR-942 (FIG. 5) as would be expected if both compounds compete for the same binding site.

A modified coregulatory peptide was identified for the biochemical assay, peptide K1 (ERRTVLQLLGNSNK; SEQ ID NO: 3). In addition to its modified sequence, the peptide K1 reagent is attached to biotin by a six carbon linker that may facilitate better accessibility of biotin with streptavidin in the peptide/receptor complex. K1 interacts with GST-mROR $\gamma$  in the absence of agonist, and therefore we also used the biochemical assay in this format in some of these experiments. In K1, the N-terminal biotin is linked to the first amino acid via 6-aminohexanoic acid (AHC), which is introduced by using Fmoc-6-aminohexanoic acid (CAS NO: 88574-06-5) during peptide synthesis. In peptide K, the N-terminal biotin is directly linked to the first amino acid without any linker.

As shown in FIG. 6, this assay could be used to characterize both an agonist (OR-12872) and an antagonist (OR-1050) independently under the same assay conditions. The methods illustrated in FIG. 5 and FIG. 6 may also be modified to allow high throughput screening of ROR $\gamma$  agonists and antagonists with the coregulatory peptide recruitment assay.

A number of analogs of T0901317 and OR-1050 were synthesized and compared in the transcriptional and biochemical assays for ROR $\gamma$  (Table 1). These findings showed that the rank order of potency of the series of related molecules was similar in the two assays. Further, several analogs of OR-942 were also synthesized, including OR-12872, an agonist with potency less than 100 nM in the biochemical assay (Table 2). The rank order of potency of these compounds was also similar in the transcriptional and biochemical assays. In one embodiment, the ROR $\gamma$  antagonist is not T0901317.

TABLE 1

The biochemical assay was carried out in the presence of mROR $\gamma$ and peptide K1.					
(Structure 1)					
				Assay EC <sub>50</sub> in micromolar at mROR $\gamma$	
	X	R1	R2	Transcriptional	Biochemical
OR-1048	OH	Br	Me	0.12	0.42
OR-1052	OH	Me	Me	0.21	0.89

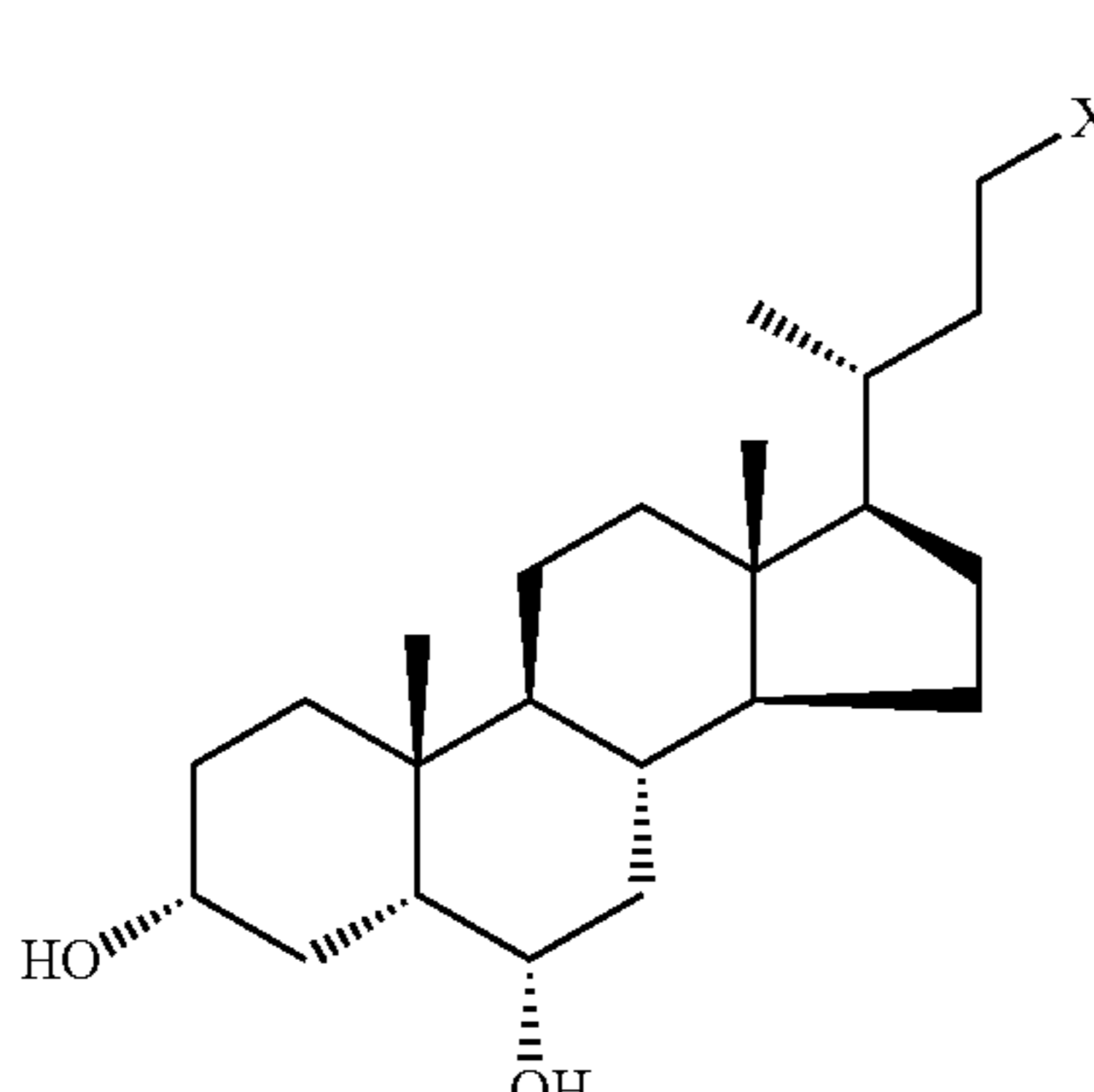
39

TABLE 1-continued

OR-1050	OH	NO <sub>2</sub>	Me	0.26	0.9
T0901317	OH	H	CF <sub>3</sub>	0.54	1.2
OR-1031	OH	H	Me	1.4	3.5
OR-1047	OH	nBu	Me	3.6	5.8
OR-1030	OMe	H	H	>20	>100
OR-1046	OH	H	CO <sub>2</sub> H	>20	>100

TABLE 2

(Structure 2)



Analogs of OR-942 (see Structure 2)

X	EC <sub>50</sub> in micromolar at mROR $\gamma$		
	Transcriptional	Biochemical	
OR-12872	CH(Me)OMe	0.44	0.052
OR-12866	C(Me) <sub>2</sub> OMe	0.56	0.14
OR-942	CO <sub>2</sub> Me	1.5	0.12
OR-12863	CH <sub>2</sub> OH	4.1	0.67
OR-12870	C(=O)Me	4.1	ND
OR-12868	C(=O)NMe <sub>2</sub>	5.2	0.56
OR-12864	CH <sub>2</sub> OMe	6	0.23
OR-12865	C(Me) <sub>2</sub> OH	8.3	0.21
OR-12871	CH(Me)OH	8.3	0.25
OR-412	CO <sub>2</sub> H	>20	>100

40

TABLE 2-continued

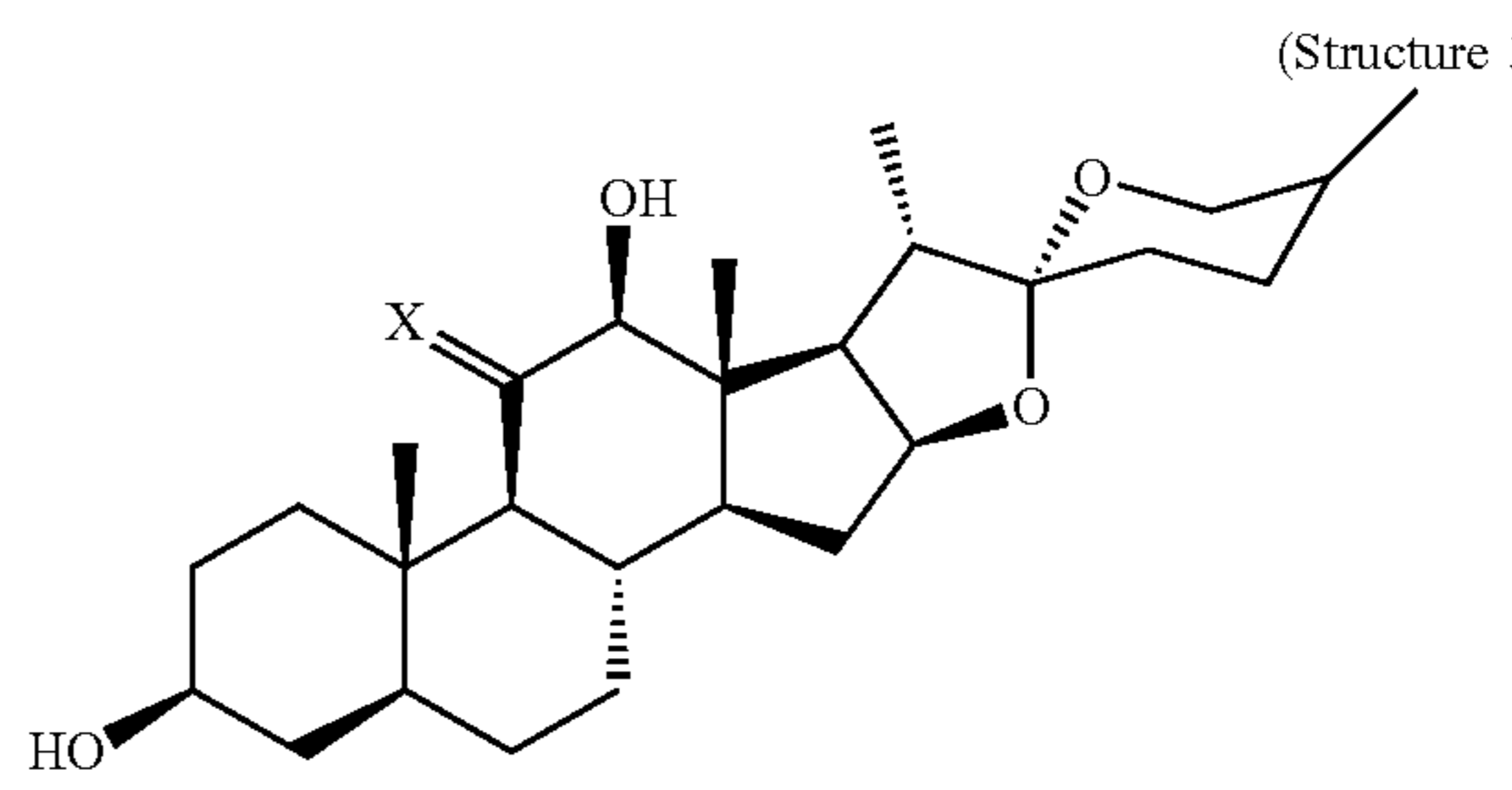
OR-12867	C(=O)NHMe	>20	ND
OR-12869	C(=O)N(c-C <sub>5</sub> H <sub>10</sub> )	>20	ND

5 Derivatives of hydoxycholeic acid methyl ester (OR-942) were evaluated in the transcriptional assay for ROR $\gamma$  and in the biochemical assay using biotinylated peptide K as the coregulatory peptide.  
 In the transcriptional assay, antagonist OR-376 (1.5  $\mu$ M) was added to reduce the basal transcriptional activity of ROR $\gamma$  thus to increase the dynamic range of the agonist effect. EC<sub>50</sub> values were estimated by fitting the data to a sigmoidal curve (GraphPad, Prism, San Diego, CA).  
 10 In the biochemical assay, the compounds induce association between the ROR $\gamma$  LBD and peptide K.  
 The EC<sub>50</sub> of the biochemical assay was determined by the concentration of compound required to elevate the baseline by 50% of estimated maximum FRET signal for the individual compound after fitting data to a sigmoidal dose response curve.

In addition, several other ROR $\gamma$  antagonists were identified, including the related terpenes OR-345 and OR-885 (rockogenin) in Table 3. The natural compound OR-13571 (11-oxo ursolic acid acetate), is illustrated in Tables 4A and 4B. Further, the structurally-distinct synthetic compound OR-2161 was also examined in ROR $\gamma$  assays.

TABLE 3

(Structure 3)



In which X = O or is absent (X = H, H)

Steroidal Antagonists	Assay EC <sub>50</sub> in micromolar		
	X	transcriptional	biochemical
OR-885	H,H	0.2	2.5
OR-345	O	5	12.7

40 The biochemical assay was carried out in the presence of peptide K and 1  $\mu$ M OR-942 to elevate the assay baseline.  
 The EC<sub>50</sub> of the biochemical assay was determined by the concentration of compound required to inhibit the OR-942 baseline by 50% after fitting data to a sigmoidal dose response curve.

TABLE 4A

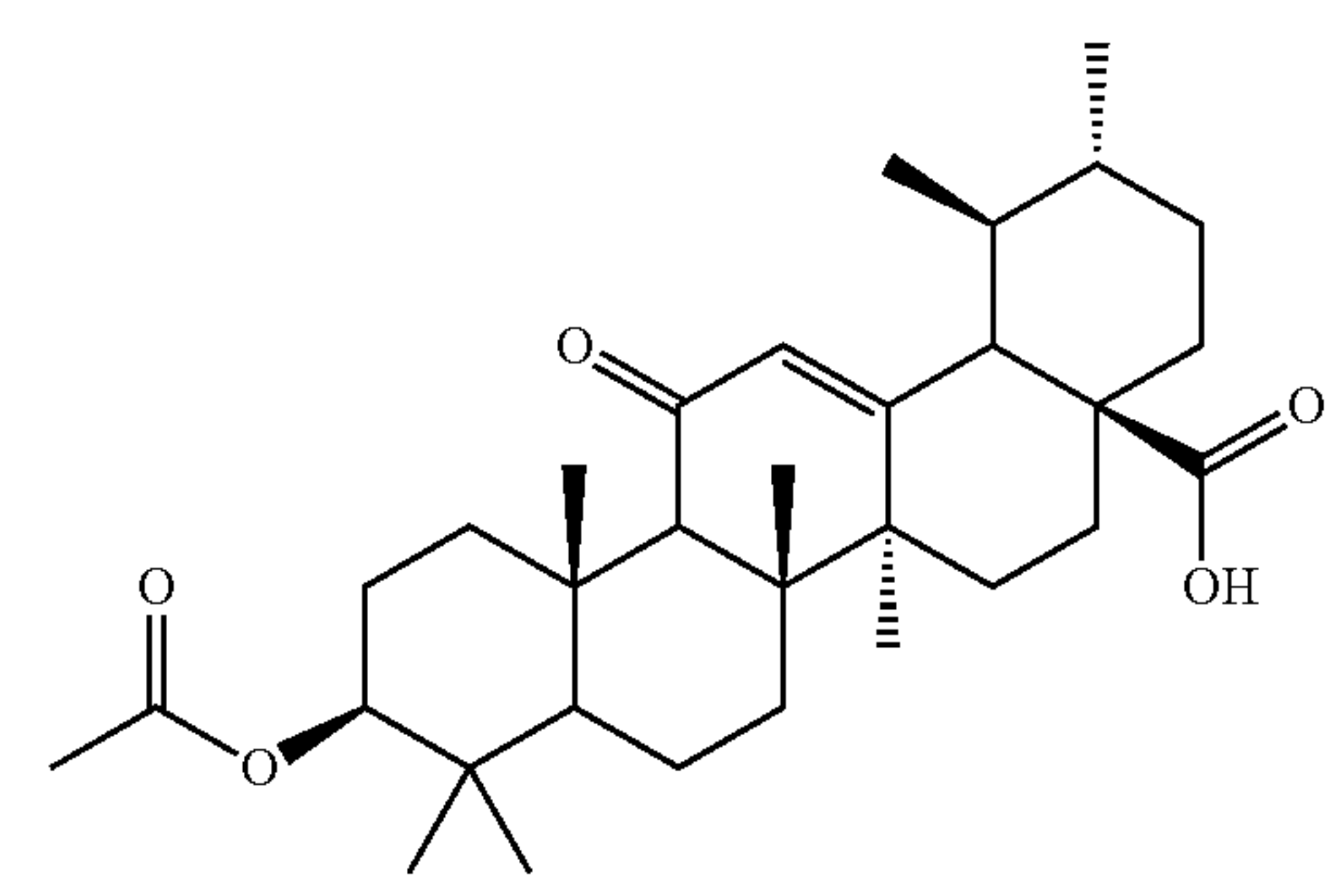
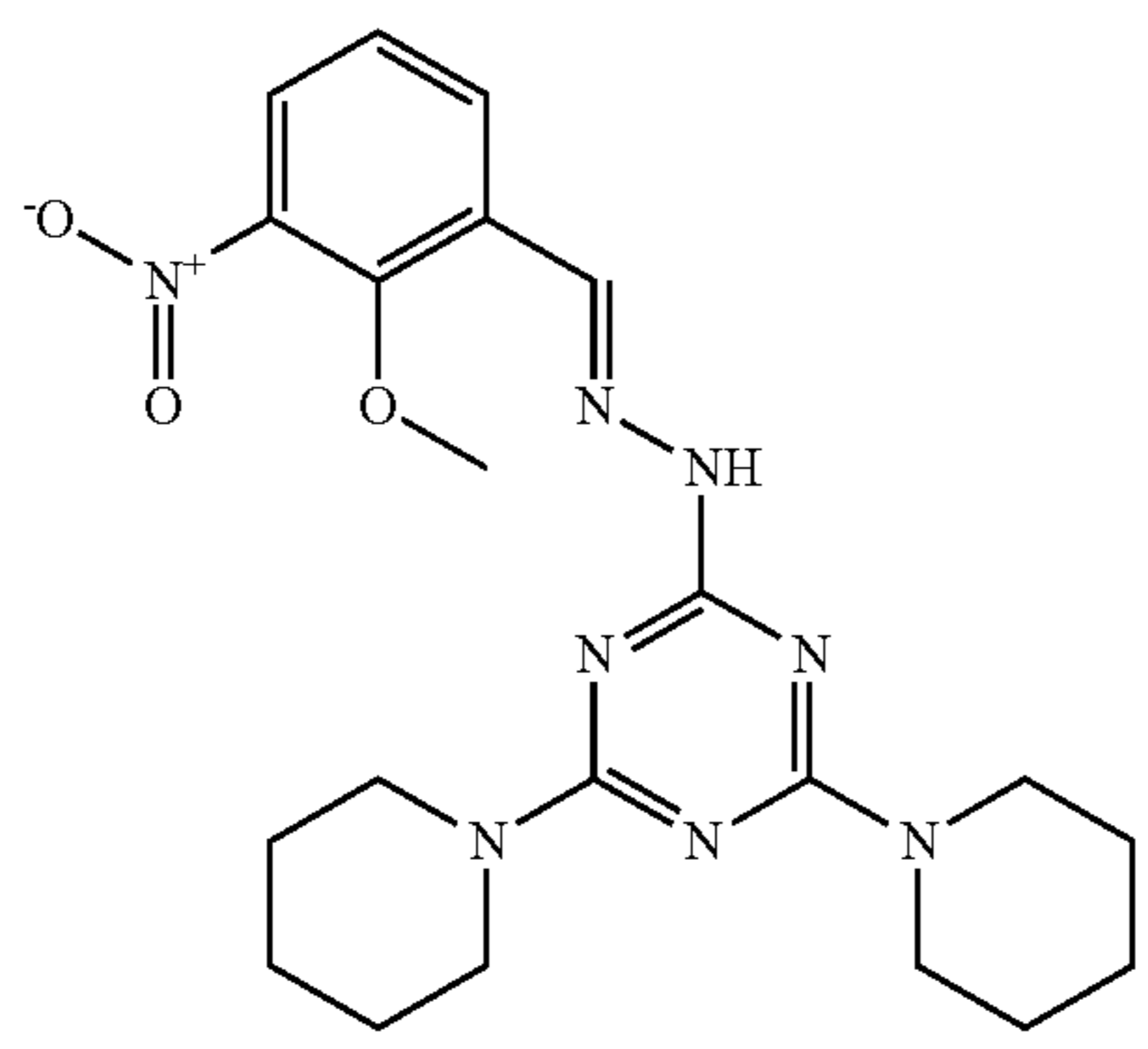
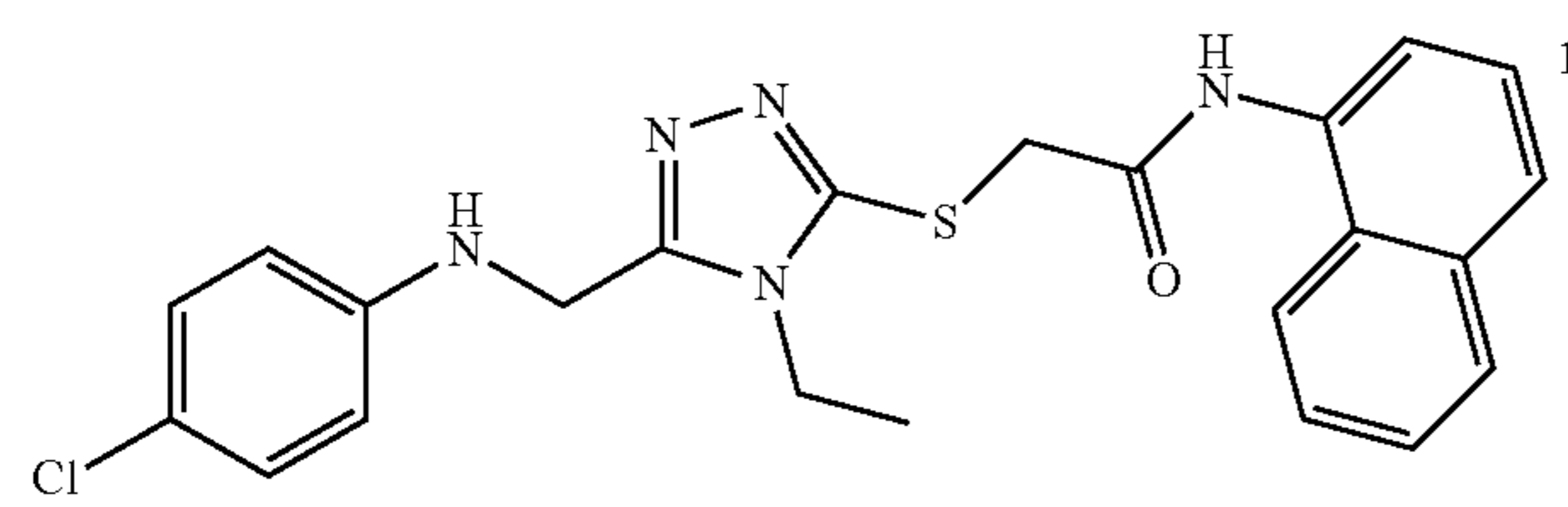
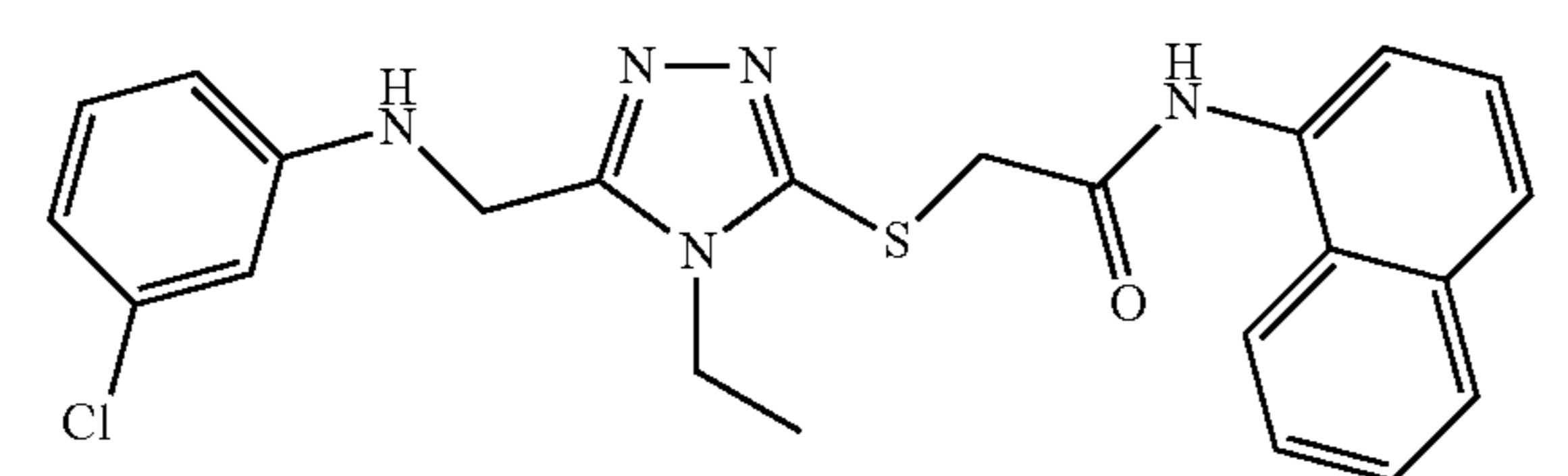
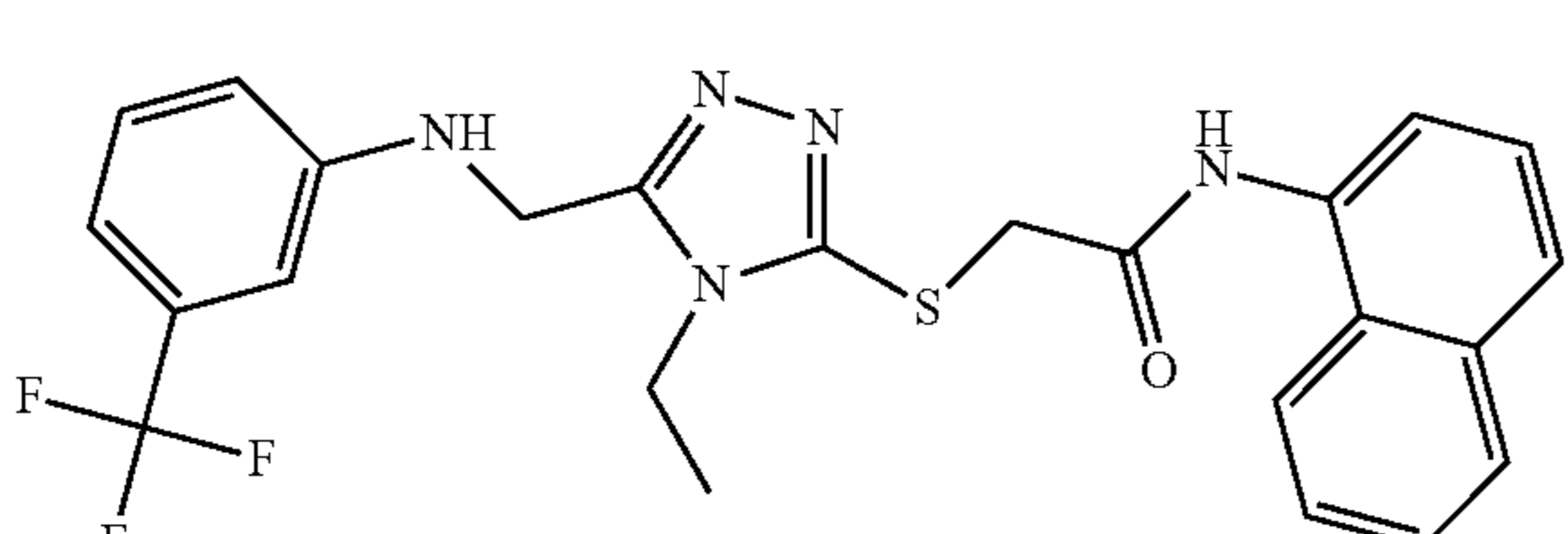
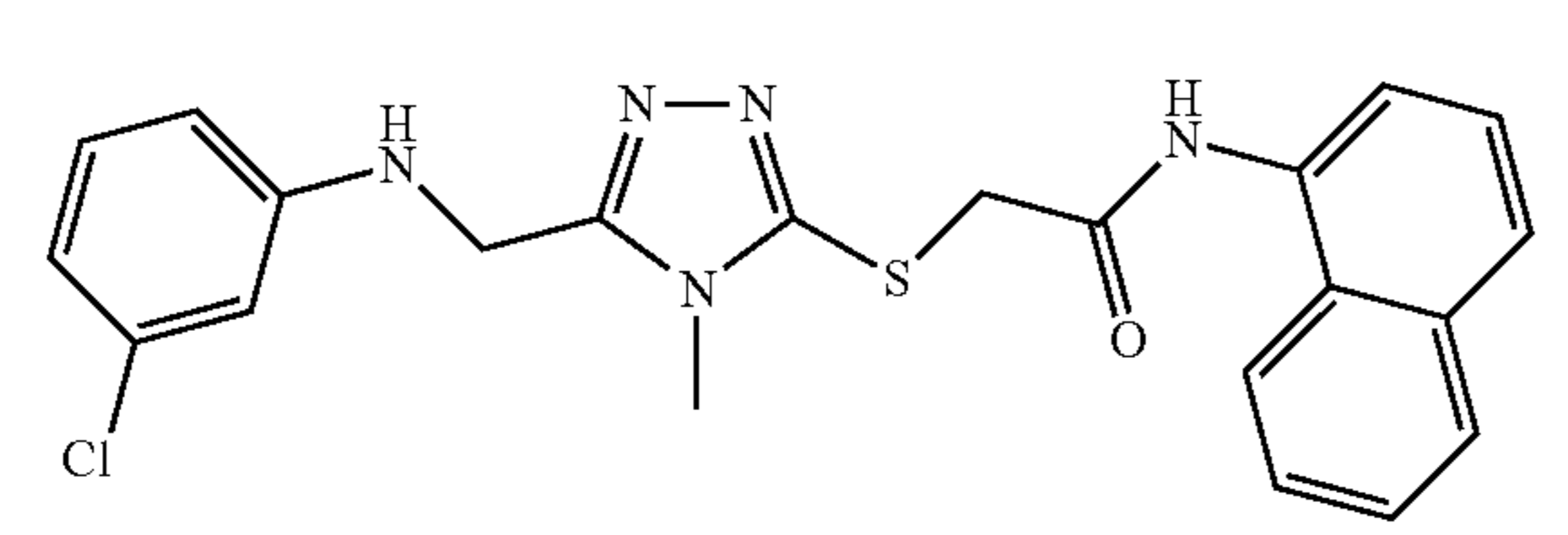
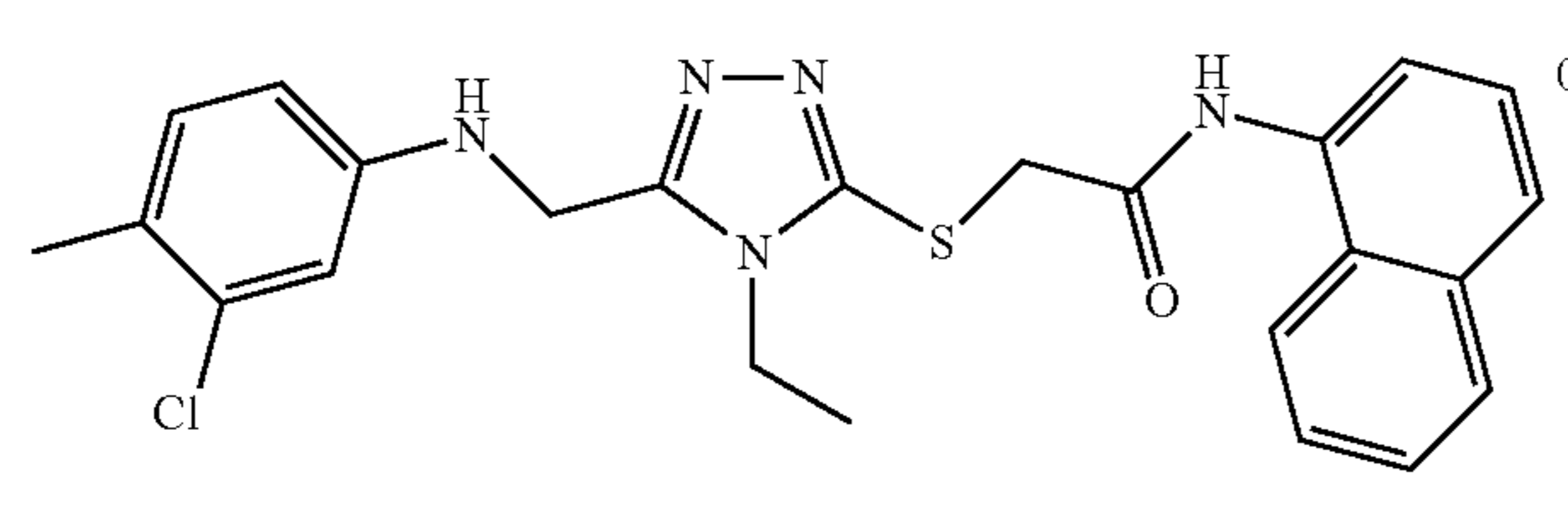
ROR $\gamma$ antagonists.			
Compound ID	Structure	EC <sub>50</sub>	EC <sub>50</sub>
		Transcriptional	Biochemical
OR-13571		0.27	1.2

TABLE 4A-continued

RORy antagonists.			
Compound ID	Structure	EC <sub>50</sub> Transcriptional	EC <sub>50</sub> Biochemical
OR-2161		0.33	4.5
OR-133008		1.46	1.61
OR-133097		1.44	1.34
OR-133099		1.04	1.33
OR-133167		3.3	3.4
OR-133171		0.49	0.44

Biochemical assays were carried out in the presence of peptide K1.

No agonist was present.

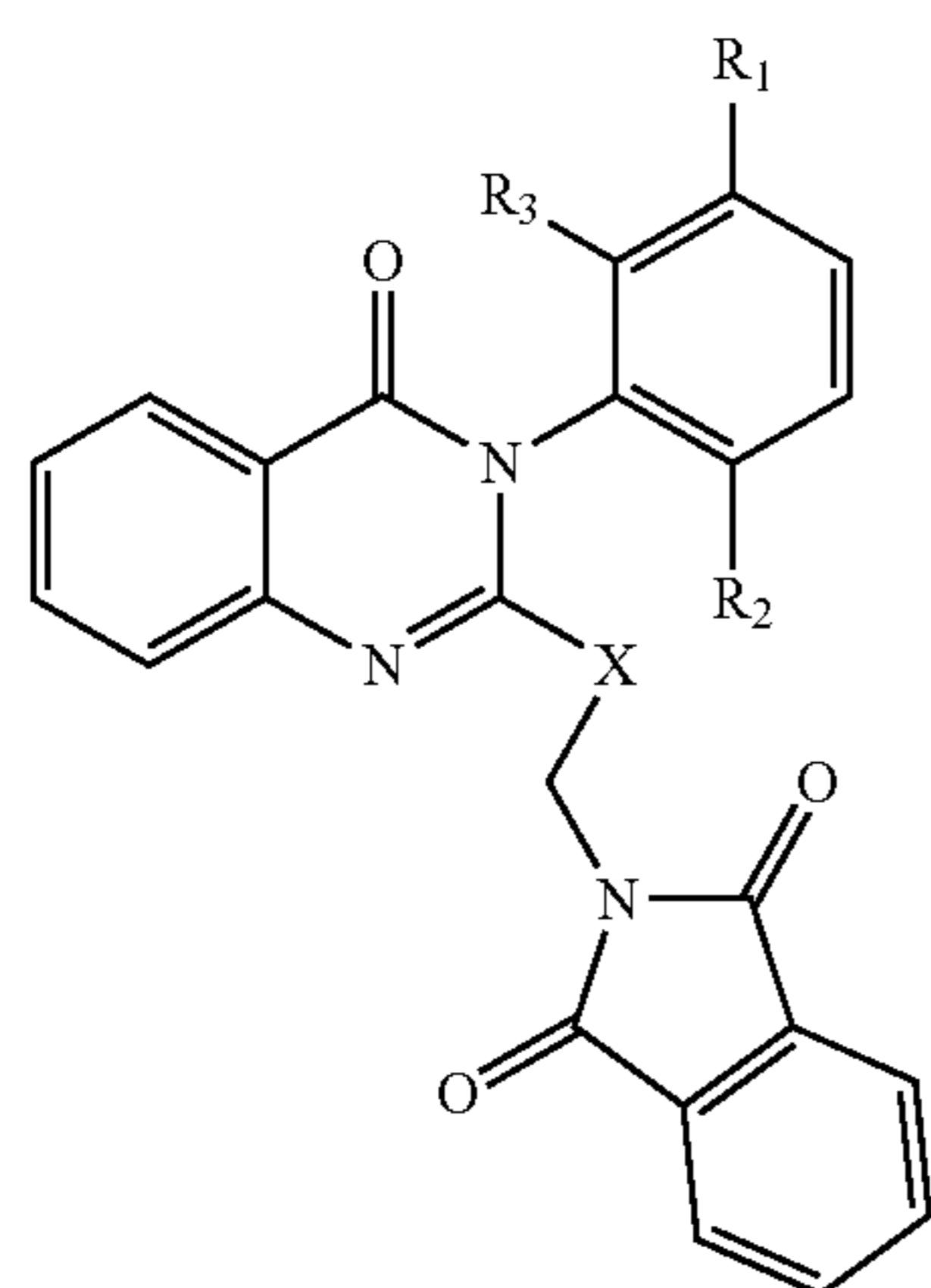
OR-13571 is 11-oxo ursolic acid acetate.

Values are medians of two or more assays.

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TABLE 4B

OR-52 and analogues of OR-52.



Structure 4

Compounds (see Structure 4)	Assay EC <sub>50</sub> in micromolar					
	X	R1	R2	R3	Transcriptional Biochemical	
OR-52	CH <sub>2</sub> CH <sub>2</sub>	CF <sub>3</sub>	H	H	5.8	3.5
OR-32286	CH <sub>2</sub>	CF <sub>3</sub>	H	H	>20	>100
OR-32268	CH <sub>2</sub> CH <sub>2</sub>	CH <sub>3</sub>	H	H	13.5	8.9
OR-32281	CH <sub>2</sub> CH <sub>2</sub>	Cl	Cl	H	3.7	5.8
OR-32288	CH <sub>2</sub> CH <sub>2</sub>	CH <sub>3</sub>	H	CH <sub>3</sub>	>20	52.2

Biochemical assays were carried out in the presence of peptide K1.  
No agonist was present.

Further, many of the compounds listed above were evaluated in a transcriptional assay for hROR $\gamma$ . As shown in Table 4C, the EC<sub>50</sub> values were comparable for most compounds tested, except for OR-885, which was significantly less potent at hROR $\gamma$ , and the analogs of OR-133008, which also were more potent at mROR $\gamma$  than at hROR $\gamma$ .

TABLE 4C

Potency of ROR $\gamma$  antagonists in a transcriptional assay of human ROR $\gamma$ .

Compound	EC <sub>50</sub> ( $\mu$ M)
OR-885	5.1
T0901317	1.1
OR-1050	0.4
OR-2161	1.0
OR-13571	0.2
OR-133008	6.8
OR-133097	5.0
OR-133099	2.4
OR-133167	9.4
OR-133171	1.7

These findings demonstrate that a range of ligands to ROR $\gamma$  may be identified by receptor screening. The example also illustrates that a series of agonists and a series of antagonists, each covering a wide range of potencies, will have consistent rank order of potencies in two mechanistically separate assays for the same target (e.g. mROR $\gamma$ ). Collectively, these findings strongly imply binding of these ligands to the ROR $\gamma$  LBD. The most potent compounds, both agonists and antagonists, are also useful for characterization of ROR $\gamma$ -mediated effects in target cells

## Example 2

It is not unusual for a single compound to interact with receptors from different subfamilies within the nuclear

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receptor superfamily (Laudet 1999). Nuclear receptor cross-reactivity is sufficiently common that it must be accounted for in a program of ligand design and characterization. An example of such a crossreactive ligand is TTNPB, which binds the retinoic acid receptors (RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$ ) from the NR1B subfamily and the farnesoid X-receptor FXR (Zavacki, Lehmann et al. 1997) in subfamily NR1H. Other examples are the estrogen receptor (NR3A) ligands diethylstilbestrol (DES), tamoxifen (TAM), and 4-hydroxytamoxifen (4-OHT) which bind the estrogen-related receptor ERR $\gamma$  (NR3B3) (Coward, Lee et al. 2001). Therefore, transcriptional assays were developed to characterize ROR $\gamma$  ligands at several of the other major nuclear receptors.

## 15 Methods

Table 5 lists details of construction of Gal4 hybrids with the following nuclear receptor LBDs. The positive control compounds for transcriptional assays (Table 6) are dexamethasone, dihydrotestosterone, 1,25 (OH)<sub>2</sub> Vitamin D<sub>3</sub>, progesterone, chenodeoxycholic acid (CDCA), TTNPB ((E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid), 9-cis retinoic acid, 17 $\beta$ -estradiol, and bezafibrate (from Sigma, St. Louis, Mo.) and T0901317, rosiglitazone and WY14643 from Cayman Chemicals. The transcriptional assays were carried out as in Example 1.

TABLE 5

The following nuclear receptor LBDs were cloned in pFA-CMV for expression as Gal4 hybrids. Receptors are described by common names found in the literature, by a Unified Receptor Nomenclature system (Laudet 1999), and by species of origin (h = human or m = mouse) and a trivial name or abbreviation. The range of amino acids included in the LBD is shown along with the cloning site in pFA-CMV. The amino acid range corresponds, in most cases, to the most common splicing isoform of the receptor. The final amino acid corresponds in each case to the C-terminal residue. Receptors were subcloned into pFA-CMV by PCR using primers containing the restriction enzyme sites shown. The methods are well known to those skilled in the art and constructs similar to these have been widely reported in the scientific literature.

Common Name of Receptor	Unified Receptor Nomenclature	Species & trivial name	Amino Acid range	cloning sites in pFACMV
glucocorticoid receptor	NR3C1	mGR	524-793	BamHI, KpnI
androgen receptor	NR3C4	hAR	646-919	BamHI, XbaI
Vitamin D receptor	NR1I1	hVDR	90-427	BamHI, HindIII
Liver X receptor alpha	NR1H3	mLXR $\alpha$	172-455	BamHI, XbaI
progesterone receptor	NR3C3	hPR	687-933	XbaI, KpnI
PPAR-gamma	NR1C3	hPPAR $\gamma$	175-477	XbaI, KpnI
farnesoid X receptor	NR1H4	hFXR	192-473	BamHI, XbaI
retinoic acid receptor alpha	NR1B1	hRAR $\alpha$	156-462	BamHI, HindIII
retinoid X receptor alpha	NR2B1	hRXR $\alpha$	203-462	EcoRI, HindIII
PPAR-alpha	NR1C1	hPPAR $\alpha$	166-468	BamHI, HindIII
PPAR-delta	NR1C2	hPPAR $\delta$	138-441	BamHI, HindIII
Estrogen receptor-alpha	NR3A1	hER $\alpha$	249-595	BamHI, KpnI

TABLE 6

Crossreactive receptor assays for ROR $\gamma$ ligands.										
Standard	Standard Ligands	ROR $\gamma$ Ligands								
		Efficacy	Potency	T0901317	OR-1050	OR-885	OR-13571	OR-12872	OR-2161	OR-133008
mGR	Dexamethasone	35X	++++	-	-	-	-	-	-	-
hAR	dihydro-testosterone	8X	++++	-	-	-	-	-	-	-
hVDR	1,25 Vitamin D3	68X	++++	-	-	-	-	-	-	-
mLXR $\alpha$	T0901317	120X	++	++	+	-	-	-	-	-
hPR	Progesterone	6X	++++	-	-	-	-	-	-	-
hPPAR $\gamma$	Rosiglitazone	50X	+++	-	-	-	-	-	-	-
hFXR	CDCA	5X	++	+	-	-	-	-	-	-
hRAR $\alpha$	TTNPB	18X	++++	-	-	-	-	-	-	-
hER $\alpha$	17 $\beta$ -estradiol	40X	++++	-	-	-	-	-	-	-
hRXR $\alpha$	9-cis-retinoic acid	180X	+++	-	-	-	-	-	-	-
hPPAR $\alpha$	WY14643	6X	*	-	-	-	-	-	-	-
hPPAR $\delta$	Bezafibrate	10X	*	-	-	-	-	-	-	-

Potency indicates EC<sub>50</sub> values in  $\mu$ M:

++++ (EC<sub>50</sub> < 0.01),

+++ (0.01-0.1),

++ (0.1-1),

+ (1-10) and

- (>10).

\*EC<sub>50</sub> values of standard ligands for PPAR $\alpha$  and PPAR $\delta$  are >>20  $\mu$ M. Efficacy = activity of standard ligand at 20  $\mu$ M. The ROR $\gamma$  ligands show no significant activity at 20  $\mu$ M in assays for PPAR $\alpha$  and PPAR $\delta$ .

## Results

Among the ROR $\gamma$  ligands identified in studies described in Example 1, one of the compounds, T0901317, was previously shown to activate LXR $\alpha$  and LXR $\beta$  (Schultz, Tu et al. 2000). A transcriptional assay for LXR $\alpha$ , using a Gal4-LXR $\alpha$  hybrid, was implemented in order to compare ROR $\gamma$  ligand potencies. In addition to LXR $\alpha$ , transcriptional assays for a number of other major nuclear receptors were developed. In Table 6, several of the ROR $\gamma$  ligands described in Example 1 were characterized against these receptors. Confirming published data on T0901317 (Schultz, Tu et al. 2000; Houck, Borchert et al. 2004), we found that T0901317 activates LXR and FXR (Table 6). At the same time, a number of other ROR $\gamma$  antagonists, such as OR-12872, OR-885, OR-13571, OR-133008, and OR-2161 were shown to be selective for ROR $\gamma$  within this receptor family.

TABLE 7

Comparative pharmacology of ROR $\gamma$ antagonists at ROR $\gamma$ and LXR $\alpha$ . Compound structures are presented in Table 1, and ROR $\gamma$ pharmacology is taken from Table 1. Activity in the LXR $\alpha$ transcriptional assay is the estimated maximum elevation of luciferase activity, based on a sigmoidal dose response curve, with respect to T0901317.				
ROR $\gamma$ antagonists	Assay EC <sub>50</sub> in micromolar at mROR $\gamma$		Transcriptional Assay of LXR $\alpha$	
	Transcriptional	Biochemical	activity	EC <sub>50</sub>
OR-1048	0.12	0.42	>80%	0.57
OR-1052	0.21	0.89	>80%	0.81
OR-1050	0.26	0.9	>80%	1.5
T0901317	0.54	1.2	100%	0.14
OR-1031	1.4	3.5	>80%	1.4
OR-1047	3.6	5.8	<5%	>20
OR-1030	>20	>100	<5%	>100
OR-1046	>20	>100	<5%	>100

In Table 7, several analogs of T0901317 were compared in transcriptional assays for ROR $\gamma$  and LXR $\alpha$ . The binding pockets of LXR $\alpha$  and LXR $\beta$  are very similar in structure and

have closely similar affinities to a range of ligands (Svensson, Ostberg et al. 2003). Hence, the LXR $\alpha$  assay is assumed to be representative of both receptors. A number of derivatives of T0901317 were synthesized. As can be seen in Table 7, compounds with a wide range of specificity were identified, including several that are more specific for ROR $\gamma$  than LXR $\alpha$ , including OR-1048, OR-1050, OR-1052 and OR-1047.

The importance of this example is two-fold. First, selective ROR $\gamma$  ligands can be identified that lack obvious cross-reactivity with other nuclear receptors. Secondly, the SAR of compounds in the T0901317 series differ between ROR $\gamma$  and LXR $\alpha$ , suggesting that ligands can be designed from this series that retain ROR $\gamma$  potency and have better separation between the two targets.

In one embodiment, the ROR $\gamma$  antagonist is at least 20-fold more potent as an ROR $\gamma$  antagonist than as an LXR agonist. In other embodiments, the ROR $\gamma$  antagonist is at least about 30-fold, 40-fold, 50-fold, 75-fold, 100-fold, 150-fold, 200-fold, 250-fold, 300-fold, 400-fold, 500-fold, 600-fold, 700-fold, 800-fold, 900-fold, or 1,000-fold more potent as an ROR $\gamma$  antagonist than as an LXR agonist.

Ligands to LXR have anti-inflammatory effects that may make them active in animal models of autoimmune disease (Zelcer and Tontonoz 2006). Recently, T0901317 was shown to inhibit the development of murine EAE (Hindinger, Hinton et al. 2006). It is not known what proportion of the therapeutic effect of T0901317 in the mouse model of EAE is due to ROR $\gamma$  antagonism as opposed to LXR activation. However, strong genetic and pharmacological data demonstrate that activation of LXR causes hypertriglyceridemia and triglyceride accumulation in liver. Specifically, in mice lacking the LXR $\alpha$  and LXR $\beta$  receptors, T0901317 has no effect on liver triglyceridemia (Schultz, Tu et al. 2000). Other LXR ligands have been tested in cynomolgus monkey and hamster (Groot, Pearce et al. 2005), and these ligands were shown to elevate serum triglycerides and serum LDL. The importance of this example is that it provides methodology to eliminate various

forms of LXR-mediated hyperlipidemic activity from the T0901317 and OR-1050 series of compounds while retaining the ability to inhibit  $T_H$ -17 differentiation.

### Example 3

The differentiation of purified naïve murine  $CD4^+$  splenic T cells into  $T_H$ -17 cells in cell culture is simulated by a combination of the cytokines TGF $\beta$  and IL-6 or inhibited by the cytokine IFN- $\gamma$  (Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006). This is not the only differentiation pathway available to naïve  $CD4^+$  T cells (FIG. 1). For example, IL-12 stimulates the differentiation of  $T_H$ 1 cells in the same system. The effects of continuous treatment with ROR $\gamma$  agonists and antagonists on  $T_H$ 1 and  $T_H$ -17 differentiation were investigated. This example demonstrates that small molecule ligands to ROR $\gamma$  regulate  $T_H$ -17 T cell differentiation. In these experiments, a  $T_H$ -17 cell was identified by cell surface staining for CD4 and intracellular staining with a labeled antibody to IL-17. In particular, we observe that antagonists to ROR $\gamma$  inhibit  $T_H$ -17 cell differentiation but have no consistent effect on the differentiation of murine  $T_H$ 1 cells (that are  $CD4^+IFN\gamma^+$  but do not express IL-17). The inhibitory effect on  $T_H$ -17 cells has been observed with four different antagonists that are each structurally distinct (that is, they are not analogs of one another). Further, supporting the conclusion that inhibition of ROR $\gamma$  activity in naïve  $CD4^+$  T cells by an ROR $\gamma$  antagonist will inhibit  $T_H$ -17 differentiation, we show that an agonist to ROR $\gamma$  reverses antagonist inhibition of  $T_H$ -17 cell differentiation and independently enhances  $T_H$ -17 cell formation. These results indicate that the ROR $\gamma$  ligands described in the example act specifically through the ROR $\gamma$  LBD in the differentiating  $T_H$ -17 cell.

#### Methods

**Antibodies and Cytokines.** The following reagents were selected for characterization of mouse lymphocytes. FITC anti-IFN $\gamma$  (XMG1.2), PE anti-CD62L (MEL-14), APC anti-CD4 (RM4-5), FITC anti-CD4 (RM4-5), PE anti-CD8 (53-6.7), APC anti-CD25 (PC61), biotinylated anti-CD25 (PC61.5), Cy5.5 anti-CD44 PE-(IM7) were from eBioscience (San Diego, Calif.). PE anti-IL-17 (TC11-18H10.1) was from BD Biosciences (San Diego, Calif.). TGF- $\beta$  and IL-6 were from Sigma. IL-12 was from PeproTech (Rocky Hill, N.J.).

**Purification of Naïve ( $CD4^+CD62L^+CD25^-$ )  $CD4$  Cells.** Spleens of 5-7 week old outbred ICR(CD-1, Harlan Labs) mice were gently minced with two 18-gauge needles in PBS buffer supplemented with 2.5 mM MgCl $_2$ , 0.5 mM CaCl $_2$  and 25 ug/ml DNase I. A single cell suspension of splenocytes was triturated with a 5-ml pipet and passed through a 40  $\mu$ m nylon mesh filter. Splenocytes were spun down at 300 $\times$ g for five minutes and resuspended in buffer containing PBS, 2% heat-inactivated FCS and 2 mM EDTA. Naïve T cells were purified from splenocytes using a  $CD4^+CD62L^+$  magnetic bead T cell Isolation Kit from Miltenyi Biotec (Auburn, Calif.) in two steps. First,  $CD4^-$  cells were removed by negative selection with biotinylated antibodies to CD8a, CD45R, CD11b, CD49b, and Ter119 (supplied by the manufacturer) and to CD25 (biotinylated PC61), added at the time of isolation. The flow through, predominantly  $CD4^+$  cells, was directly labeled with anti-CD62L microbeads and enriched on a magnetic column to about 93% purity, as determined by staining for CD4, CD62L, and CD25.

**Stimulation and Culture of  $T_H$ -17 Cells.** Purified  $CD4^+CD62L^+$  T cells were plated in 24-well plates with  $2-4 \times 10^5$

cells in 700  $\mu$ l volume of RPMI 1640 media containing 10% heat-inactivated FCS, 100 IU penicillin, 100  $\mu$ g/ml streptomycin, 1 $\times$ non-essential amino acids, 1 mM pyruvate, 2 mM glutamine, and 50  $\mu$ M  $\beta$ -mercaptoethanol (Veldhoen, Hocking et al. 2006). T cells were stimulated with exogenous cytokines and anti-CD3/anti-CD28 conjugated Dynabeads (Dynal Biotech, Oslo) at a 1:1 ratio of beads to cells. Exogenous cytokines used were: 4 ng/ml TGF- $\beta$ , 20 ng/ml of IL-6, or 10 ng/ml IL-12. ROR $\gamma$  agonist and antagonist ligands, dissolved in DMSO, were added directly to cell cultures, to a final concentration of 0.01%. Cytokines and ROR $\gamma$  ligands were added immediately after cell plating.

**Cell Staining and Flow Cytometry.** To determine intracellular cytokine production, exogenous cytokines were removed by centrifugation and cultures treated with 500 ng/ml phorbol 12,13 dibutyrate (PdBu), 500 ng/ml ionomycin, and 1  $\mu$ g/ml brefeldin A for 4-6 hours to stimulate cytokine production while preventing secretion as described (Veldhoen, Hocking et al. 2006) and washed again. Cells were then fixed, permeabilized and stained for intracellular cytokines using commercial reagents (eBioscience, San Diego). Cells were also stained for the cell surface markers CD4 and CD8. Flow cytometry analysis was performed on a FACSCalibur (Becton Dickinson) and the data were analyzed using FlowJo Software (Tree Star, Inc).

**ELISA for IL-17.** ELISA kits for mouse IL-17 and human IL-17 were from eBioscience (San Diego, Calif.). ELISA assays were performed following the manufacturer's protocol. Certain samples were diluted 20-fold to avoid maximization of enzymatic signals. For mouse IL-22 ELISA, antibodies and standards were from Antigenix America (Huntington Station, N.Y.) and accessory components were from eBioscience. The IL-22 assay protocol was similar to that of the IL-17 assays.

#### Results

Mouse  $T_H$ -17 cells can be induced to differentiate from naïve  $CD4^+$  T cells in the presence of TGF $\beta$  and IL-6 (Veldhoen, Hocking et al. 2006). At the same time,  $T_H$ 1 differentiation is suppressed. Critical steps in this protocol are: (i) purification of naïve  $CD4^+$  cells to remove potentially inhibitory activated T cells, such as  $T_H$ 1 cells, or T regulatory (Treg) cells, (ii) activation of T cells by cross-linking CD3 and CD28, and (iii) incubation with TGF $\beta$  and IL-6 to induce  $T_H$ -17 differentiation. Protocols for  $T_H$ -17 differentiation have been reported by several laboratories (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006). The percentage of differentiated cells is determined by intracellular staining for IL-17 and IFN- $\gamma$ , markers of  $T_H$ -17 and  $T_H$ 1 cells respectively (Park, Li et al. 2005). A  $T_H$ -17 differentiation assay provides a cell-based model to demonstrate ROR $\gamma$  antagonist function. We predicted that OR-1050, OR-885, OR-13571, and OR-2161 should inhibit  $T_H$ -17 differentiation and/or IL-17 release and that this inhibitory effect could be reversed by the potent ROR $\gamma$  agonist OR-12872.

Naïve  $CD4^+$  T cells were isolated from splenocytes of 5-8 wk old CD-1 mice and  $CD4^+CD62L^+$  cells with approximately 93% purity selected with magnetic beads. We estimated that the population of Tregs (Itoh, Takahashi et al. 1999) in the purified cells, as estimated by the percentage of  $CD25^+CD44^+$  cells, was about 1-1.5% as compared to 5% in total splenocytes. IL-12 treatment of the naïve T cells stimulates  $T_H$ 1 differentiation as shown by the high percentage of IFN $\gamma$  positive cells, while the combination of TGF $\beta$ 1 and IL-6 induced the formation of 4% IL-17-producing cells (Table 8). As reported, the  $T_H$ 1 (IFN $\gamma^+$ ) and  $T_H$ -17 (IL-17 $^+$ ) phenotypes were mutually exclusive (see also FIG. 8).

Further, both IL-6 and TGF $\beta$  were required to fully induce the T<sub>H</sub>-17 phenotype as reported in the scientific literature (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006). Finally, we observed that the IL-17<sup>+</sup> cells were >97% CD4<sup>+</sup>.

TABLE 8

IL-17- or IFN $\gamma$ -producing cells are differentiated from naïve CD4 cells in the presence of exogenous cytokines. Cells are stained for IL-17 or IFN- $\gamma$ and quantitated by FACS analysis. Data are shown as mean $\pm$ SD of the percentage of IL-17 or IFN $\gamma$ -producing cells in total cell populations after 5 days in culture. Less than 0.02% of total cells appear to express both cytokines.		
	IL-17 (%)	IFN $\gamma$ (%)
No cytokine	0.21 $\pm$ 0.00	2.50 $\pm$ 0.40
IL-12 (10 ng/ml)	0.09 $\pm$ 0.01	39.6 $\pm$ 2.44
TGF $\beta$ 1 (4 ng/ml)	0.21 $\pm$ 0.02	0.32 $\pm$ 0.05
IL-6 (20 ng/ml)	1.20 $\pm$ 0.04	3.42 $\pm$ 0.08
TGF $\beta$ 1 + IL-6	4.53 $\pm$ 0.18	2.14 $\pm$ 0.18

For pharmacology studies, we added either agonist or antagonist to ROR $\gamma$  at the initiation of naïve CD4<sup>+</sup> T cell cultures described above. We found that OR-1050 significantly inhibited T<sub>H</sub>-17 cell differentiation in a dose-dependent manner while the potent agonist OR-12872 was stimulatory (see FIG. 7).

As expected from receptor pharmacology studies, OR-12872 was able to reverse the effect of OR-1050, consistent with the conclusion that both compounds act through the same target, i.e., ROR $\gamma$  (FIG. 8). In this experiment, the agonist OR-12872 alone elevated T<sub>H</sub>-17 frequency by 60%, but in the presence of OR-1050 the agonist caused a 400% percent increase from the lower baseline produced by OR-1050, precisely the behavior expected if agonist and antagonist bind the same target. The figure represents individual observations, and the average of duplicate observations is presented in Table 9. Neither 3  $\mu$ M OR-1050 nor 3  $\mu$ M OR-12872 had an effect on the frequency of T<sub>H</sub>1 cell differentiation in the presence of IL-12. The findings show that OR-1050 is unlikely to block T<sub>H</sub>-17 cell formation through a non-specific cytotoxic effect for two reasons: it does not inhibit T<sub>H</sub>1 differentiation and the blockage of T<sub>H</sub>-17 differentiation is reversible by an ROR $\gamma$  agonist.

TABLE 9

Naïve CD4 <sup>+</sup> T cells were induced to differentiate with 4 ng/ml TGF $\beta$ and 20 ng/ml IL-6 (T <sub>H</sub> -17 protocol) or 10 ng/ml IL-12 (Th-1 protocol). After addition of compound, the final DMSO concentration in the culture wells was 0.01%. Cytokine positive cells were determined as in the methods for this example. Duplicate cell cultures were counted and averaged.			
Compound	Cell Frequency (%)		
	T <sub>H</sub> -17 Protocol		Th-1 Protocol
	IL-17 <sup>+</sup>	IFN- $\gamma$ <sup>+</sup>	IFN- $\gamma$ <sup>+</sup>
DMSO	2.32%	1.60%	36.3%
3 $\mu$ M OR-1050	0.44%	1.42%	37.4%
3 $\mu$ M OR-12872	3.91%	1.37%	41.4%
OR-1050 + OR-12872	1.79%	1.47%	36.6%

To provide additional confirmation that T<sub>H</sub>-17 cell differentiation can be pharmacologically-regulated through inhibition of ROR $\gamma$  function, we tested the effects of the antagonist OR-885 and its reversal by OR-12872. FIG. 9 demonstrates that OR-885 blocks the differentiation of

T<sub>H</sub>-17 cells and that this effect is reversed by OR-12872. The results of this experiment are summarized in Table 10. It appears that there may be a small secondary effect of ligand on Th-1 cell levels. Overall, however, the ROR $\gamma$  ligands did not have a consistent effect on Th-1 cell frequency in these studies.

TABLE 10

Naïve CD4 <sup>+</sup> T cells were induced to differentiate with 4 ng/ml TGF $\beta$ and 20 ng/ml IL-6 (T <sub>H</sub> -17 protocol) or 10 ng/ml IL-12 (Th-1 protocol). After addition of compound, the final DMSO concentration in the culture wells was 0.01%. Cytokine positive cells were determined as in the methods for this example. Duplicate cell cultures were counted.			
Compound	Cell Frequency (%)		
	T <sub>H</sub> -17 Protocol		Th-1 Protocol
	IL-17 <sup>+</sup>	IFN- $\gamma$ <sup>+</sup>	IFN- $\gamma$ <sup>+</sup>
DMSO vehicle	2.46%	0.71%	29.7%
3 $\mu$ M OR-885	0.52%	1.45%	34.2%
1 $\mu$ M OR-12872	3.96%	0.65%	30.8%
OR-885 + OR-12872	1.33%	1.11%	36.2%

Finally, OR-13571 exerted a similar effect on T<sub>H</sub>-17 differentiation as OR-885 and OR-1050. The effect of 3  $\mu$ M OR-1050 was compared to that of 3  $\mu$ M OR-13571 in Table 11.

TABLE 11

Studies were performed as in Table 9.		
Compound	Cell Frequency (%)	
	Th-17 Differentiation	
	IL-17 <sup>+</sup>	IFN- $\gamma$ <sup>+</sup>
DMSO vehicle	3.81%	1.03%
3 $\mu$ M OR-1050	1.47%	1.09%
3 $\mu$ M OR-13571	1.42%	0.93%

The data show that OR-13571 also inhibits T<sub>H</sub>-17 differentiation at a concentration of 3  $\mu$ M. In summary, this example provides very strong pharmacological evidence that regulation of ROR $\gamma$  activity by small molecule ligands can control cell fate. Antagonists with 3 distinct structures were shown to have a similar effect on T<sub>H</sub>-17 differentiation and two of these were reversed by a specific ROR $\gamma$  agonist, OR-12872. It is probable that the ROR $\gamma$  ligands will regulate T<sub>H</sub>-17 function in other settings described in the literature, including by measurement of IL-17 release into surrounding medium and by culture of cells from lymph nodes of animals treated with antigen that provokes autoimmune disease (Murphy, Langrish et al. 2003; Langrish, Chen et al. 2005; Mangan, Harrington et al. 2006). This hypothesis is addressed in Example 6.

We also investigated whether the concomitant release of IL-17 that is expected to take place with T<sub>H</sub>-17 differentiation is pharmacologically regulated by ROR $\gamma$  ligands. ROR $\gamma$  antagonists at 3  $\mu$ M were added as naïve CD4<sup>+</sup> T cells were induced to differentiate into T<sub>H</sub>-17 cells, in the presence or absence of OR-12872. In this study, all four ROR $\gamma$  antagonists tested block IL-17 release (FIG. 10). Significantly, the presence of OR-12872, the ROR $\gamma$  agonist, reverses the effect of each of the antagonists, indicating that the compounds are acting in an ROR $\gamma$ -specific manner. We hypothesize that the



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very marked reduction in IL-17 levels involved simultaneous inhibition of  $T_H$ -17 differentiation, as shown above, and suppression of IL-17 release.

## Example 4

This example provides data suggesting that pharmacological repression of ROR $\gamma$  will not necessarily inhibit thymic function in vivo. The induction of liver triglycerides by OR-1050 in C57BL/6 mice following oral dosing was investigated. In addition, the effect of compound treatment in the same animals on the number and distribution of the major thymocyte populations was analyzed. The objective was to show evidence of bioavailability through elevation of liver triglycerides (through LXR), and at the same time to

## Methods

Following six days of twice-a-day dosing with corn oil vehicle or 50 mg/kg OR-1050 in corn oil, mice were sacrificed and the thymus and liver removed and weighed. A small piece of the liver (~200 mg) was weighed and extracted to determine triglyceride content. Glycerol was liberated from liver triglycerides by hydrolysis in base. The liver fragment was incubated overnight at 55° C. in 0.35 mls of ethanolic KOH (2 parts EtOH: 1 part 30% KOH) in a closed tube. A solution of 50% ethanol in water was added

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4% formalin in PBS and stored at 4° C. for 48 hours. Before analytical flow cytometry was carried out (FACS Calibur, Becton Dickinson), Caltag counting beads (from Invitrogen) were added to allow absolute quantitation of cell number.

5 Thymus subpopulations were determined after gating for live cells. As a positive control for thymic involution, two animals were also treated once with 10 mg/kg dexamethasone two days before sacrifice. Dexamethasone reproducibly induces rapid apoptosis of DP thymocytes and reduction of thymic mass (Chmielewski, Drupt et al. 2000; Zubkova, Mostowski et al. 2005).

## Results

Table 12 shows that liver triglyceride content (in mg of triglyceride/gram liver) is markedly elevated by OR-1050 as reported for other LXR agonists (Schultz, Tu et al. 2000; Beyer, Schmidt et al. 2004). In contrast, the number and fraction of major thymocyte subpopulations was not significantly affected. As expected, dexamethasone caused a virtually total depletion of DP thymocytes.

20 As shown above, OR-1050 is approximately 5-fold more potent in repression of ROR $\gamma$  transcription than it is in induction of LXR transcription. Thus it is likely that ROR $\gamma$  activity in mice treated as above was significantly repressed. This example demonstrates that pharmacological dosing of an ROR $\gamma$  antagonist does not necessarily cause thymic atrophy or loss of thymocytes following an intermediate period of dosing.

TABLE 12

Effects of OR-1050 on liver triglycerides and thymocyte distribution. C57BL/6 mice were treated by vehicle only (corn oil) or OR-1050 (100 mg/kg) for 7 days, or by dexamethasone (Dex, 12.5 mg/kg) once 2 days before necropsy. Corn oil and OR-1050 (50 mg/kg) were dosed twice per day by gavage. Liver triglycerides are reported as mg triglyceride/gram liver wet weight. One animal from the OR-1050 group had a very low frequency of DP thymocytes (<1% of control) and was excluded from this analysis.							
Group	Liver triglycerides (mg/g)	Thymus weight ( $\mu$ g)	Total cells per spleen ( $\times 10^{-6}$ )			Frequency of DP (% of total cells)	
			DP	SP4	SP8		
Vehicle	6	34.7 $\pm$ 5.7	71.3 $\pm$ 12.0	58.7 $\pm$ 24.7	6.3 $\pm$ 1.7	2.0 $\pm$ 0.4	52.6 $\pm$ 8.4
Dex	2	35.3 $\pm$ 8.2	49.7 $\pm$ 1.2	1.0 $\pm$ 0.4	3.9 $\pm$ 0.1	1.2 $\pm$ 0.1	4.2 $\pm$ 2.9
OR-1050	5	119.5 $\pm$ 18.8	76.1 $\pm$ 5.8	61.5 $\pm$ 10.0	8.1 $\pm$ 1.6	2.5 $\pm$ 0.5	56.7 $\pm$ 3.1

to bring the volume of each tube to 1.0 ml. The solution was cleared of debris by centrifugation, and the resulting supernatant increased to 1.2 ml with further addition of 50% ethanol. Aqueous 1 M MgCl<sub>2</sub> (215  $\mu$ l) was added to 200  $\mu$ l of the supernatant, and the mixture was cleared by centrifugation again after standing 10 min on ice. Glycerol levels were measured with the Sigma Free Glycerol Reagent (Sigma-Aldrich, St. Louis, Mo.) according to the manufacturer's instructions. The triglyceride concentration (in milligrams per gram of liver wet weight) was determined by assuming average molecular weight of 1000 daltons for each molecule of triglyceride.

In addition, the total number and the fraction of the major thymocyte subpopulations including CD4<sup>+</sup>CD8<sup>+</sup> (DP), CD4<sup>+</sup>CD8<sup>-</sup> (SP4<sup>+</sup>) and CD4<sup>-</sup>CD8<sup>+</sup> (SP8<sup>+</sup>) were measured by cell surface staining followed by FACS analysis. The thymus was dissected, weighed, and gently pressed against a wire screen (200 mesh USA standard test sieve, Newark Wire Cloth Company) with plunger of a 5 ml plastic syringe. The volume of cells was adjusted to 8 mls. Debris from the thymus was allowed to settle, and a 200  $\mu$ l aliquot of cells was incubated with fluorescent antibodies to CD4 and CD8 for 15 minutes at room temperature. The cells were fixed in

## Example 5

This example provides a demonstration that an ROR $\gamma$  antagonist may inhibit the development of EAE in a mouse model.

## Methods

8-10 week old, female C57BL/6 mice or SJL/J mice were purchased from Harlan laboratories (Harlan, Indianapolis, Ind.) and housed in a specific pathogen free (SPF) animal facility for one week before the start of the studies. Mice were immunized with peptide antigens intradermally at the dorsal flanks on day 0. Peptide antigens are either 150  $\mu$ g MOG<sub>35-55</sub> (MEVGWYRSPFSRVVHLYRNGK (SEQ ID NO: 19), AnaSpec, San Jose, Calif.) per C57BL/6 mouse or 75  $\mu$ g PLP<sub>139-151</sub> (HSLGKWLGHDPKF (SEQ ID NO: 20), Bio-Synthesis, Lewisville, Tex.) per SJL/J mouse. Peptides were dissolved in PBS and emulsified with an equal volume of Complete Freund's Adjuvant (CFA) containing 4 mg/ml of H37RA *M. tuberculosis* (Chondrex, Redmond, Wash.). To ensure induction of reliable EAE, 200 ng of pertussis toxin (List Biologicals, Campbell, Calif.) was given by intravenous injection (i.v.) at day 0 and 2 (Cua, Sherlock et al. 2003; Zhang, Gran et al. 2003). Animals were treated with

drug beginning one day before peptide injection. Animals were randomized into groups for treatment by weight, so that the average weight of each group was similar. The groups were not segregated by cage. Instead, members of all groups were represented in every cage in order to minimize environmental effects on the outcome of the study. In the C57BL/6 study, OR-1050 was suspended in corn oil by sonication at 6 mg/mL or 20 mg/mL and animals were dosed twice daily by gavage with 2.5 mls/kg body weight of corn oil for each dose. In one SJL/J study (FIG. 11D & E), OR-1050 was dissolved in dimethylformamide at 200 mg/ml, further diluted into three parts of HRC-6, loaded into an osmotic pump (Alzet 2002) and implanted in the interscapular region under anesthesia 2-3 days after peptide immunization. The estimated daily dose was 30 mg/kg in this study. In another SJL/J study (see FIG. 11F), mice were treated once daily by gavage with 100 mg/kg OR-1050 solubilized in the synthetic vehicle HRC-6.

Clinical symptoms are scored by a visual inspection of behavior, along the following scale (Papenfuss, Rogers et al. 2004): 1) Limp tail or waddling gait; 2) waddling gait with tail limpness; 3) partial hind limb paralysis; 4) complete hind limb paralysis; 5) moribund state, leading to death by EAE. At severity level 4 or 5 the animals were sacrificed to prevent further suffering. Animals were weighed daily during the treatment period as a secondary measure of the disease course. As a comparative measure for drug treatment, the median day of onset was compared between groups. Statistical analysis was by the Kruskal-Wallis test, a non-parametric test for comparison of multiple groups. On study day 27, remaining animals were euthanized, and brain and spinal cord fixed, embedded in paraffin, and H&E sections analyzed for inflammatory infiltration. For the studies of inflammation, several sections of spinal cord (7 to 8) were characterized by an experienced veterinary pathologist in a blinded fashion. Inflammation in each section was evaluated visually using a semi-quantitative scale based on the overall severity of the sample (e.g., Severity: 0, not present; 1, slight; 2, mild; 3, moderate; 4, severe).

#### Results

C57BL/6 mice were treated with OR-1050 by oral gavage twice a day for a total dose of 30 mg/kg or 100 mg/kg for 25 days. Mice were injected with a peptide that induces autoimmune disease in this mouse strain one day after the start of dosing. The progress of the disease in the vehicle and high dose groups is illustrated in FIG. 11A and FIG. 11B, where exposure to OR-1050 both inhibits progress of the disease and weight loss due to the onset of symptoms. FIG. 11C shows that inflammatory infiltration in the spinal cord was reduced by treatment with OR-1050 in these animals. The onset of disease in the three groups as a function of number of days since peptide immunization is presented in FIG. 12. The median day of onset of symptoms (clinical score 1 or greater) was day 18 for the corn oil vehicle control, day 16 for the 30 mg/kg group, and day 26 for the 100 mg/kg treated group (FIG. 12A). The median day of onset for untreated animals was day 15 (not shown). The difference in day of onset between the vehicle and the 100 mg/kg treated animals was statistically significant ( $p < 0.05$ ). This example shows that an ROR $\gamma$  antagonist is able to delay the onset of EAE. The surviving animals at day 27 showed a difference in the severity of inflammation in spinal cord between vehicle and high dose treated animals. The statistical significance was based on a rank order test ( $p < 0.07$ , Mann-Whitney).

In a second EAE mouse study, SJL/J mice were immunized with PLP<sub>139-151</sub> and OR-1050 was dosed by osmotic

pump to achieve more stable compound delivery starting at day 4. OR-1050 also reduced paralytic severity (FIG. 11D) and onset (FIG. 11E). In each of these studies, there was a significant difference in severity or weight on several days, as indicated in the graphs (FIG. 8A, B&D). Using a more rigorous statistical analysis, we calculated the area under the severity curve for each of the mice in FIG. 11D, and compared the mean values  $\pm$  sem for vehicle ( $14.5 \pm 1.6$ ) and treated ( $5.3 \pm 2.7$ ) groups. The differences were significant ( $p < 0.01$ ) based on the Mann-Whitney rank order test (GraphPad Prism, San Diego). Furthermore, as shown in FIG. 11E, the day of onset was significantly delayed in the treated SJL/J mice.

Since OR-1050 inhibits T<sub>H</sub>-17 cells in ex vivo culture, we also investigated whether T<sub>H</sub>-17 cell frequency was affected in OR-1050-treated animals. SJL/J female mice ( $n=9$ /group) were treated with 100 mg/kg OR-1050 in HRC-6 beginning two days before immunization with PLP (as described above) and continuing until 8 days after immunization. Animals were sacrificed on day 9, and T<sub>H</sub>-17 cells analyzed from pooled axillary, brachial, and inguinal lymph nodes from each animal. We found a 35% reduction in T<sub>H</sub>-17 frequency in lymph nodes of SJL/J mice (FIG. 11F) as a fraction of the total CD4<sup>+</sup> cell population. The change in T<sub>H</sub>-17 frequency with OR-1050 treatment was statistically significant.

In a second study on C57BL/6 mice (FIG. 11G), we observed a similar effect on splenic T<sub>H</sub>-17 cells in C57BL/6 mice (29% inhibition,  $p < 0.05$ ,  $n=8-10$ ) with no change in T<sub>H</sub>1 cells. C57BL/6 female mice were immunized with 150  $\mu$ g MOG<sub>33-55</sub> on study day 0. Mice were treated with either OR-1050 at 100 mg/kg or with vehicle (HRC-6) by oral gavage for 9 days starting day 0. Splenocytes were collected at Day 9 and stained for T<sub>H</sub>-17 (CD4<sup>+</sup>CD8<sup>-</sup>IL17<sup>+</sup>) and T<sub>H</sub>1 (CD4<sup>+</sup>CD8<sup>-</sup>IFN $\gamma$ <sup>+</sup>) cells. Statistics were performed by Student's t-test. Collectively, the data suggest that OR-1050 inhibits the expansion of T<sub>H</sub>-17 cells in response to peptide antigen.

In a separate study related to these findings, we demonstrated that OR-1050 is orally bioavailable (FIG. 12B). Pharmacokinetics of OR-1050 in CD-1 mice was analyzed at independent bioanalytical laboratory. Male CD-1 mice were treated via oral gavage with OR-1050 at 5 mg/kg in HRC-6. Blood was collected via retro-orbital puncture in tubes containing sodium heparin anticoagulant at 0, 5, 15, and 30 min, and at 1, 2, 4, 6, 8 and 24 hr after oral administration. The concentration of OR-1050 in plasma was determined using an HPLC/MS/MS method. The peak concentration of OR-1050 ( $\sim 100$  ng/ml) suggests that a higher dosage (50 mg/kg) will give blood levels, 1  $\mu$ g/ml or  $\sim 2$   $\mu$ M, that is higher than the EC<sub>50</sub> for OR-1050 in transcriptional assays in in vivo pharmacological studies.

#### Example 6

That ROR $\gamma$  antagonists inhibit the differentiation of T<sub>H</sub>-17 is consistent with the phenotype of the ROR $\gamma$ <sup>-/-</sup> mouse, which lacks T<sub>H</sub>-17 cells and where naive CD4<sup>+</sup> T cells resist differentiate to T<sub>H</sub>-17 in the presence of IL-6 and TGF $\beta$  (Ivanov, McKenzie et al. 2006). The question of whether the reactivation of memory T<sub>H</sub>-17 cells can be blocked by ROR $\gamma$  ligands is not addressed by the ROR $\gamma$ <sup>-/-</sup> mouse phenotype, since memory T<sub>H</sub>-17 cells are unable to differentiate from naive CD4<sup>+</sup> T cells. To address the issue, we cultured lymph node cells from mice previously immunized with the myelin-derived peptides PLP<sub>139-151</sub>, MOG<sub>35-55</sub>, or complete Freund's adjuvant (CFA) alone. Memory T

cells are known to proliferate in response to cognate peptide in these cultures, and our controls showed that stimulation of  $T_H$ -17 proliferation and IL-17 release were dependent on prior immunization with peptide, consistent with a memory T cell response (FIG. 13A). Thus, LN cultures from CFA-immunized mice were insensitive to PLP in 3 day culture, whereas LN cultures from mice immunized with PLP (emulsified in CFA) showed a ~10-fold increase in IL-17 release and a concomitant two-fold elevation in the frequency of  $T_H$ -17 cells in response to peptide (not shown). These data show that IL-17 induction is closely linked to the peptide-dependent increase in  $T_H$ -17 cells and therefore provides a convenient method of characterizing compound effects on Th-17 cells. Furthermore, OR-885 inhibited the PLP-stimulated release of IL-17. The correlation between Th-17 cell number and IL-17 release in the presence of agonist and antagonist was further confirmed (FIGS. 13B and 13C), and the antagonist OR-885 was shown to block both the induction of  $T_H$ -17 cells (as a percentage of total live cells) and IL-17 release.

Overall, cultured lymph node cells that are stimulated with IL-23 and peptide antigens provide a model system to examine IL-17 production *ex vivo* (FIG. 13D). We analyzed a variety of ROR $\gamma$  ligands for their ability to regulate IL-17 (FIG. 13E). Four different classes of ROR $\gamma$  antagonists tested all inhibit IL-17 release, while the agonist is able to activate IL-17. A dose-response assay suggested that the antagonist OR-885 inhibits IL-17 production with an EC<sub>50</sub> value of approximately 0.2  $\mu$ M (FIG. 13F). Each point is the average of duplicate measurements of IL-17 release in a single well. This potency is consistent with that measured in the CHO cell transcription assay. To further confirm that the activity of compounds was ROR $\gamma$ -dependent in this system, we showed that the agonist OR-12872 reverses the suppressive effects of OR-885 on IL-17 release in lymph node cultures (FIG. 14).

The proinflammatory cytokine IL-22 is also expressed in Th-17 cells. We characterized IL-22 release from lymph node cell cultures and found that like IL-17, IL-22 release is induced by both peptide antigen and IL-23, and that the effect of both was more than additive. In addition, IL-22 seems to have 30-50 fold higher levels than IL-17. IL-22 production is also inhibited by the ROR $\gamma$  antagonists OR-885 and OR-1050 and enhanced by the agonist OR-12872 at a concentration of 3  $\mu$ M each in LN cultures, similar to findings with the IL-17 assays. This is illustrated in FIG. 15.

Regulation of Human IL-17 Release in Cultured PBMCs. We also investigated whether human IL-17 is subject to regulation by ROR $\gamma$  ligands. It has proven difficult to differentiate Th-17 cells from naïve human T cells under the same conditions as mouse (Zheng, Danilenko et al. 2007), although there are now several very recent reports that this can be done (Acosta-Rodriguez, Napolitani et al. 2007; Annunziato, Cosmi et al. 2007; Wilson, Boniface et al. 2007). Since mitogenic stimuli such as ConA or PMA will stimulate IL-17 expression in memory T cells in human PBMC (Yao, Painter et al. 1995; Shin, Benbernou et al. 1999), we used this protocol for an initial investigation of IL-17 regulation by ROR $\gamma$  ligands. Frozen human peripheral blood mononuclear cells (PBMCs from Stem Cell Technologies, Vancouver) were thawed and cultured for 3 days with 1  $\mu$ g/ml Con A and 3  $\mu$ M compounds in lymphocyte culture medium (RPMI 1640 media containing 10% heat-inactivated FCS, 100 IU penicillin, 100  $\mu$ g/ml streptomycin, 1 $\times$ non-essential amino acids, 1 mM pyruvate, 2 mM glutamine, and 50  $\mu$ M  $\beta$ -mercaptoethanol). Culture media were

analyzed by hIL-17 ELISA (mean $\pm$ sem, n=4; ANOVA with Bonferroni post test). In FIG. 16A, the effects of 3  $\mu$ M OR-885 and 3  $\mu$ M OR-12872 on IL-17 release from PBMCs were compared. The effect of OR-885 was partially reversible by OR-12872. FIG. 16B shows that stimulated IL-17 release into culture medium by ConA is inhibited by OR-13571 and OR-1050. The possibility that inhibition mediated by these two compounds is due to cytotoxicity was addressed by co-treatment with the ROR $\gamma$  agonist OR-12872. OR-12872 alone does not significantly alter IL-17 release but it does partly reverse the effects of OR-1050 and OR-13571, suggesting that both compounds act specifically through ROR $\gamma$ .

In a separate study, T-cell blasts were generated from human PBMC according to methods previously described (Hoeve, Savage et al. 2006). Frozen T-cell blasts were thawed and cultured for 18 hours in a 24-well plate with 1 $\times$ 10<sup>6</sup> cells per well in 700  $\mu$ L of lymphocyte culture medium. Phorbol 12,13-dibutyrate (500 ng/ml), ionomycin (500 ng/ml) and compounds (3  $\mu$ M) were added at the beginning of the culture. Culture media were collected and analyzed for hIL-17 level by ELISA. The results of treatment with OR-1050 and OR-13571 are shown in FIG. 16C.

#### Example 7

Methods. 8 to 10-week old male DBA/1 (DBA/10IaHsd, Harlan Laboratories) mice are housed in a specific pathogen free (SPF) animal facility. Arthritis is induced by two injections of collagen subcutaneously in the base of the tail. The initial injection (on day 0) uses bovine type II collagen (2 mg/ml from Chondrex, Redmond, Wash.) emulsified in equal volume of CFA containing 4 mg/ml of *M. tuberculosis* (Chondrex). The CII booster injection on Day 29 is emulsified in incomplete Freund's adjuvant (IFA). Each animal receives 0.1 ml of emulsion by subcutaneous/intradermal injection in the tail 2 to 3 cm from the body of the mouse. The booster injection site is in the vicinity of but different from the initial injection site and closer to the body of the animal. OR-1050 was formulated in HRC-6 as above. On weekdays, the animals received two doses (a.m. and p.m.) of HRC-6 or 50 mg/kg OR-1050 p.o. (2.5 mls/kg). On weekends, a single dose of 100 mg/kg was administered (5 mls/kg).

The mice were observed daily for clinical symptoms of CIA based on the following qualitative scale. Each paw was examined individually and scored. Grade 0, normal; grade 1, mild but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; grade 2, moderate redness and swelling of ankle or wrist; grade 3, severe redness and swelling of the entire paw including digits; grade 4, maximally inflamed limb with involvement of multiple joints. To estimate cumulative disease severity for each animal, an area under the curve score was calculated for each animal by totaling the sum of the daily hind paw measurements between days 24 and 48. Because of the difficulty of adequately distinguishing multiple gradations of change in the front paws, scoring of the hind paw only was used in FIG. 17.

Results. We investigated the effect of the ROR $\gamma$  antagonist OR-1050 on the course of collagen-induced arthritis in mouse, where  $T_H$ -17 cells are clearly involved (Courtenay, Dallman et al. 1980; Sato, Suematsu et al. 2006). Animals were treated with OR-1050 (p.o.) starting three days before immunization with collagen type II. The disease phenotype is first clearly visible about one week after the second

injection of CII (at day 29), and the disease course is partly suppressed in the OR-1050-treated animals when compared to vehicle controls (FIG. 17A). Based on the difference in overall disease severity determined in the hind limbs of the animals (FIG. 17B), the response shows a trend ( $p < 0.09$ ) towards a statistically significant effect.

#### Example 8

The present invention provides for the discovery of novel small molecule antagonists to ROR $\gamma$  that are useful in the treatment of autoimmune disease and other conditions involving the inhibition of T<sub>H</sub>-17 cell activity. The steps required to identify a clinical candidate can be outlined briefly. (1) Novel compounds derived from diverse screening libraries, or by directed chemical synthesis from known hits, are tested for activity in transcriptional and biochemical assays for ROR $\gamma$ . (2) Hits from this primary screening are then characterized for specificity against a panel of other nuclear receptors as described above. (3) Candidate lead compounds for more extensive animal studies are selected from these hits on the basis of potency and selectivity. A candidate lead is expected to inhibit murine T<sub>H</sub>-17 cell formation in culture. The candidate lead molecules are tested for other desirable properties such as bioavailability and metabolic stability that enable therapeutic use of the compound in animal models of disease. (4) One or more lead ROR $\gamma$  antagonists are tested in animal models of EAE, CIA and IBD which are known to respond to other agents that inhibit T<sub>H</sub>-17 function. If active in one or more of these models, the compounds are tested for general immunotoxicity, effects on thymic function and overall immune response, and on general aspects of toxicity such as liver triglyceride content and serum lipids known to be affected by ligands to several nuclear receptors. (5) Such leads are further examined for properties that would prevent use in humans. These include potential for drug-drug interactions and general toxicity in rodents and at least one other higher species. If problems in the areas of drug-drug interactions or toxicity are identified, compounds may be further modified by synthetic methods, rescreened, and reevaluated to reduce these side effects. Other features that may be optimized at this stage are bioavailability, chemical stability, and ease of manufacture. (6) One or more candidate clinical compounds are selected for safety studies leading to Investigative New Drug status granted by the FDA. This step enables human clinical trials that will provide evidence for safety and therapeutic utility. The execution of steps two and beyond is well understood by practitioners of the art of drug discovery. The validation of ROR $\gamma$  antagonist effects in T<sub>H</sub>-17 cells provides the scientific rationale for undertaking drug development.

As another example, the invention envisages modification of hit or lead molecules to generate lead candidates. Examples of hits useful for further medicinal synthetic work are OR-1050 or OR-133171 described above. Analogs of these affect potency at ROR $\gamma$  in a logical manner. Significantly, a representative member of the OR-133171 series was shown to have no measurable transcriptional activity at LXR $\alpha$ . More potent compounds may be modified by parallel synthetic modification of the core scaffolds of these two compounds in pharmacological assays. These assays may identify compounds that increase potency. Other assays for metabolic stability and bioavailability may be introduced in order to identify a useful lead compound. The invention also provides for the discovery of alternative compound series to those indicated above, in case these fail to provide molecules

with characteristics suitable for commercial development, by screening additional small molecule drug-like compound libraries.

A drug from the ROR $\gamma$  antagonist class will ideally have good oral bioavailability and a half-life of four hours or more, enabling once or twice-daily administration. Nuclear receptor ligands have commonly given rise to orally bioavailable drugs; OR-1050 is an example of an orally-bioavailable ROR $\gamma$  antagonist characterized in these studies. The advantages of an orally bioavailable drug are: (i) direct oral administration is feasible; (ii) unlike injectable biologics, which may have an extremely long half life, a small molecule drug with reasonable half-life can be withdrawn if necessary to limit side effects and (iii) small molecule drugs are readily manufactured. Such compounds may also be selected for clinical development by potency in animal models of EAE, CIA and IBD and by parallel in vivo studies of compound safety. Examples of useful models for investigation of ROR $\gamma$  antagonist effects on EAE, in addition to the C57BL/6 mouse immunized with the MOG<sub>35-55</sub> peptide, are female SJL/J mice induced with the myelin proteolipid peptide (PLP<sub>139-151</sub>) (Papenfuss, Rogers et al. 2004) and the Lewis rat (Bolton, O'Neill et al. 1997). EAE may also be induced by adoptive transfer of cultured, antigen-specific T<sub>H</sub>-17 cells from previously immunized mice (Langrish, Chen et al. 2005). CIA is induced by injection of type 2 collagen (Courtenay, Dallman et al. 1980) and this model is recognized to be IL-23 and T<sub>H</sub>-17 cell dependent (Murphy, Langrish et al. 2003; Lubberts, Koenders et al. 2004). Mouse models of IBD are now recognized to have a significant involvement of T<sub>H</sub>-17 cells (Yen, Cheung et al. 2006; Zhang, Zheng et al. 2006; Elson, Cong et al. 2007). Certain genetic models such as the IL-10<sup>-/-</sup> mouse (Yen, Cheung et al. 2006) have physiological relevance for understanding disease and have been shown to require IL-23 for disease induction, but the disease course itself is prolonged and highly variable in these animals, rendering them unfavorable for pharmacological studies of reasonable duration. On the other hand, transfer of T cell populations with a reduced component of T regulatory cells to immunodeficient mice will cause disease within about 10 weeks (Powrie and Uhlir 2004), a time frame suitable for pharmacological testing. Disease causation in the T cell transfer model is also dependent on IL-23 (Yen, Cheung et al. 2006). Older mouse IBD models that induce damage to the intestinal lining, such as by treatment with dextran sodium sulfate (DSS) in drinking water (Spahn, Herbst et al. 2002), are acceptable disease models and may also include a significant contribution by T<sub>H</sub>-17 cells.

While the invention has been described with reference to specific examples, this description is not meant to limit the kind of small molecule drug that may be used as part of practicing this invention.

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## SEQUENCE LISTING

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 1 5 10 15

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tctcccaaaa ccaaaaggct tccgctgact agggcacatc tgacagaagt ggaatcaagg    180
ctagaagac  tggaacagct atttctactg atttttctc  gagaagacct tgacatgatt    240
ttgaaaatgg attctttaca ggatataaaa gcattgtaa  caggattatt tgtacaagat    300
aatgtgaata aagatgccgt cacagataga ttggcttcag tggagactga tatgcctcta    360
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ctacggcagc gcaccaacct cttttcacgg gaggagggtga ccagctacca gaggaagtca    180
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tgcagcgagc tcatcagctc catatthgac ttttcccact tctcagcgc cctgtgtttt    480
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catctctgca agactcatcg acaaggcctc ctagccaagc tgccacccaa aggaaaactc    660
cggagcctgt gcagccaaca tgtggaaaag ctgcagatct tccagcacct ccacccatc    720
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 20           25           30
Lys Asn Asn Trp Glu Cys Arg Tyr Ser Pro Lys Thr Lys Arg Ser Pro
 35           40           45
Leu Thr Arg Ala His Leu Thr Glu Val Glu Ser Arg Leu Glu Arg Leu
 50           55           60
Glu Gln Leu Phe Leu Leu Ile Phe Pro Arg Glu Asp Leu Asp Met Ile
 65           70           75           80
Leu Lys Met Asp Ser Leu Gln Asp Ile Lys Ala Leu Leu Thr Gly Leu
 85           90           95
Phe Val Gln Asp Asn Val Asn Lys Asp Ala Val Thr Asp Arg Leu Ala
 100          105          110
Ser Val Glu Thr Asp Met Pro Leu Thr Leu Arg Gln His Arg Ile Ser
 115          120          125
Ala Thr Ser Ser Ser Glu Glu Ser Ser Asn Lys Gly Gln Arg Gln Leu
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Thr Val Ser Pro
145

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&lt;223&gt; OTHER INFORMATION: Peptide linker

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&lt;212&gt; TYPE: PRT

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&lt;223&gt; OTHER INFORMATION: Xaa = any amino acid

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 20           25           30
Cys Gln Leu Arg Leu Glu Asp Leu Leu Arg Gln Arg Thr Asn Leu Phe
 35           40           45
Ser Arg Glu Glu Val Thr Ser Tyr Gln Arg Lys Ser Met Trp Glu Met
 50           55           60
Trp Glu Arg Cys Ala His His Leu Thr Glu Ala Ile Gln Tyr Val Val
 65           70           75           80
Glu Phe Ala Lys Arg Leu Ser Gly Phe Met Glu Leu Cys Gln Asn Asp
 85           90           95
Gln Ile Ile Leu Leu Thr Ala Gly Ala Met Glu Val Val Leu Val Arg
 100          105          110

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Met Cys Arg Ala Tyr Asn Ala Asn Asn His Thr Val Phe Phe Glu Gly  
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Lys Tyr Gly Gly Val Glu Leu Phe Arg Ala Leu Gly Cys Ser Glu Leu  
 130 135 140

Ile Ser Ser Ile Phe Asp Phe Ser His Phe Leu Ser Ala Leu Cys Phe  
 145 150 155 160

Ser Glu Asp Glu Ile Ala Leu Tyr Thr Ala Leu Val Leu Ile Asn Ala  
 165 170 175

Asn Arg Pro Gly Leu Gln Glu Lys Arg Arg Val Glu His Leu Gln Tyr  
 180 185 190

Asn Leu Glu Leu Ala Phe His His His Leu Cys Lys Thr His Arg Gln  
 195 200 205

Gly Leu Leu Ala Lys Leu Pro Pro Lys Gly Lys Leu Arg Ser Leu Cys  
 210 215 220

Ser Gln His Val Glu Lys Leu Gln Ile Phe Gln His Leu His Pro Ile  
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Val Val Gln Ala Ala Phe Xaa Pro Leu Tyr Lys Glu Leu Phe Ser Thr  
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 aaccgcacgg tcttttttga aggcaaatac ggtggcatgg agctgttccg agccttgggc 420  
 tgcagcgagc tcatcagctc catctttgac ttctcccact ccctaagtgc cttgcacttt 480  
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 ctccaagaga aaaggaaagt agaacagctg cagtacaatc tggagctggc ctttcatcat 600  
 catctctgca agactcatcg ccaaagcatc ctggcaaagc tgccaccca ggggaagctt 660  
 cggagcctgt gtagccagca tgtggaaagg ctgcagatct tccagcacct ccaccccatc 720  
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 Cys Gln Leu Arg Leu Glu Asp Leu Leu Arg Gln Arg Ser Asn Ile Phe  
 35 40 45  
 Ser Arg Glu Glu Val Thr Gly Tyr Gln Arg Lys Ser Met Trp Glu Met  
 50 55 60  
 Trp Glu Arg Cys Ala His His Leu Thr Glu Ala Ile Gln Tyr Val Val  
 65 70 75 80  
 Glu Phe Ala Lys Arg Leu Ser Gly Phe Met Glu Leu Cys Gln Asn Asp  
 85 90 95  
 Gln Ile Val Leu Leu Lys Ala Gly Ala Met Glu Val Val Leu Val Arg  
 100 105 110  
 Met Cys Arg Ala Tyr Asn Ala Asp Asn Arg Thr Val Phe Phe Glu Gly  
 115 120 125  
 Lys Tyr Gly Gly Met Glu Leu Phe Arg Ala Leu Gly Cys Ser Glu Leu  
 130 135 140  
 Ile Ser Ser Ile Phe Asp Phe Ser His Ser Leu Ser Ala Leu His Phe  
 145 150 155 160  
 Ser Glu Asp Glu Ile Ala Leu Tyr Thr Ala Leu Val Leu Ile Asn Ala  
 165 170 175  
 His Arg Pro Gly Leu Gln Glu Lys Arg Lys Val Glu Gln Leu Gln Tyr  
 180 185 190  
 Asn Leu Glu Leu Ala Phe His His His Leu Cys Lys Thr His Arg Gln  
 195 200 205  
 Ser Ile Leu Ala Lys Leu Pro Pro Lys Gly Lys Leu Arg Ser Leu Cys  
 210 215 220  
 Ser Gln His Val Glu Arg Leu Gln Ile Phe Gln His Leu His Pro Ile  
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tggcgaaaca aaaagtttga attgggtttg gagtttccca atcttcctta ttatattgat    180
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acatatttaa atggtgatca tgtaacccat cctgacttca tgttgatga cgctcttgat    480
gttgttttat acatggaccg aatgtgctg gatgcgttcc caaaattagt ttgttttaaa    540
aaacgtattg aagctatccc acaaattgat aagtacttga aatccagcaa gtatatagca    600
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gacagcccag atctgggtac cgggtggtggc tccggtgatg acgacgacaa gagtcccatg    180
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<400> SEQUENCE: 19

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 1           5           10

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What is claimed is:

1. A method of inhibiting  $T_H$ -17 cell differentiation, function or activity in an individual afflicted with multiple sclerosis, psoriasis, Crohn's disease, asthma, rheumatoid arthritis, uveitis or psoriatic arthritis, comprising delivering to said individual an effective amount of a small organic molecule antagonist of ROR $\gamma$  such that said antagonist

60 contacts cells of said individual in vivo, wherein said small organic molecule antagonist has a molecular weight of less than about 600 daltons, wherein said small organic molecule to be delivered has the ability to inhibit transcription from a Gal4 construct comprising a human ROR $\gamma$  ligand binding domain in a Chinese hamster ovary host cell, wherein its 65 potency of inhibition of the transcription, measured as EC<sub>50</sub>,

is less than that of T0901317, and wherein said small organic molecule antagonist of ROR $\gamma$  is not T0901317.

2. The method of claim 1, wherein said T<sub>H</sub>-17 cell function or activity is release of a cytokine.

3. The method of claim 2, wherein said cytokine is interleukin-17 or interleukin-22.

4. The method of claim 1, wherein the ROR $\gamma$  antagonist is orally administered.

5. The method of claim 1 wherein the ROR $\gamma$  antagonist is locally administered.

6. The method of claim 1 wherein said effective amount of a ROR $\gamma$  antagonist is administered on a daily basis without resulting in weight loss or hypertriglyceridemia.

7. A method of treating multiple sclerosis, psoriasis, Crohn's disease, asthma, rheumatoid arthritis, uveitis or psoriatic arthritis, in an individual, comprising identifying an individual in need of such treatment, and delivering to said individual an effective amount of a small organic molecule antagonist of ROR $\gamma$  such that said antagonist contacts cells of said individual in vivo, wherein said small organic molecule antagonist has a molecular weight of less than about 600 daltons, wherein said small organic molecule to be delivered has the ability to inhibit transcription from a Gal4 construct comprising a human ROR $\gamma$  ligand binding domain in a Chinese hamster ovary host, wherein its potency of inhibition of the transcription, measured as EC<sub>50</sub>, is less than that of T0901317, and wherein said small organic molecule antagonist of ROR $\gamma$  is not T0901317.

8. The method of claim 7, wherein the ROR $\gamma$  antagonist is orally administered.

9. The method of claim 7, wherein said ROR $\gamma$  antagonist is locally administered.

10. The method of claim 7, wherein said effective amount of ROR $\gamma$  antagonist is administered on a daily basis without resulting in weight loss or hypertriglyceridemia.

11. The method of claim 1, wherein said ROR $\gamma$  antagonist does not activate an unrelated nuclear receptor selected from the group consisting of human FXR, human PPAR- $\alpha$ , human PPAR- $\delta$ , human PPAR- $\gamma$ , mouse GR, human AR, mouse LXR- $\alpha$ , human RAR $\alpha$  or human PR, wherein said ROR $\gamma$  antagonist has an EC50 at said unrelated nuclear receptor as determined by induction of transcription from a Gal4 construct comprising a nuclear receptor binding domain of said unrelated nuclear receptor in said Chinese hamster ovary host cell that is greater than the EC<sub>50</sub> of said antagonist at human ROR $\gamma$ .

12. The method of claim 7, wherein said ROR $\gamma$  antagonist does not activate an unrelated nuclear receptor selected from the group consisting of human FXR, human PPAR- $\alpha$ , human PPAR- $\delta$ , human PPAR- $\gamma$ , mouse GR, human AR, mouse LXR- $\alpha$ , human RAR $\alpha$  or human PR, wherein said ROR $\gamma$  antagonist has an EC50 at said unrelated nuclear

receptor as determined by induction of transcription from a Gal4 construct comprising a nuclear receptor binding domain of said unrelated nuclear receptor in said Chinese hamster ovary host cell that is greater than the EC<sub>50</sub> of said antagonist at human ROR $\gamma$ .

13. The method of claim 1, wherein the potency of inhibition of said ROR $\gamma$  antagonist is at least that of OR-1050.

14. The method of claim 1, wherein the potency of inhibition of said ROR $\gamma$  antagonist is at least that of OR-2161.

15. The method of claim 1, wherein the potency of inhibition of said ROR $\gamma$  antagonist is at least that of OR-13571.

16. The method of claim 4, wherein said ROR $\gamma$  antagonist is administered in a dosage form selected from the group consisting of a tablet, pill, dragee, capsule, liquid, gel, slurry and suspension.

17. The method of claim 1, wherein said ROR $\gamma$  antagonist is topically administered.

18. The method of claim 17, wherein said ROR $\gamma$  antagonist is administered in a dosage form selected from an ointment, gel, cream, paste, salve, gel, cream, lotion and transdermal patch.

19. The method of claim 1, wherein said ROR $\gamma$  antagonist is administered by inhalation.

20. The method of claim 1, wherein said ROR $\gamma$  antagonist is administered as an aerosol spray.

21. The method of claim 7, wherein the potency of inhibition of said ROR $\gamma$  antagonist is at least that of OR-1050.

22. The method of claim 7, wherein the potency of inhibition of said ROR $\gamma$  antagonist is at least that of OR-2161.

23. The method of claim 7, wherein the potency of inhibition of said ROR $\gamma$  antagonist is at least that of OR-13571.

24. The method of claim 8, wherein said ROR $\gamma$  antagonist is administered in a dosage form selected from the group consisting of a tablet, pill, dragee, capsule, liquid, gel, slurry and suspension.

25. The method of claim 7, wherein said ROR $\gamma$  antagonist is topically administered.

26. The method of claim 25, wherein said ROR $\gamma$  antagonist is administered in a dosage form selected from an ointment, gel, cream, paste, salve, gel, cream, lotion and transdermal patch.

27. The method of claim 7, wherein said ROR $\gamma$  antagonist is administered by inhalation.

28. The method of claim 27, wherein said ROR $\gamma$  antagonist is administered as an aerosol spray.

\* \* \* \* \*